Genetic Diversity and Population Structure of the Cosmopolitan Blue Shark *Prionace glauca* (Linnaeus, 1758) in the Atlantic Ocean

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Orientador
Doutora Ana Veríssimo, Investigadora Pós-doutoral, CIBIO, FCUP

"Fundos do mar fluctuando maravilhas,
Que há milhões de anos crias, mar, ou tragas,
Submersas bases coralinas de ilhas
Abrindo em flor à flor das glaucas vagas,
Bordados de algas e subtis rendilhas,
Irreais cidades de animais-flores-fragas"

José Régio

"É arrepiante pensar que uma simples mudança de direcção poderia representar a perda das alternativas biológicas que nos tornam verdadeiramente humanos. Claro que, nesse caso, nunca teríamos vindo a saber que nos faltava alguma coisa."

António Damásio

"Plus je compare, plus je suis."

Denis Reynaud

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Abstract

The blue shark (*Prionace glauca*) is an oceanic and epipelagic shark with a cosmopolitan distribution, and one of the most abundant carcharhinid sharks. This great traveler can perform long migrations up to 7176 km within ocean basins. Blue sharks are also the main by-catch species of swordfish and tuna fisheries in the Atlantic Ocean, and have shown recent declines in abundance in the North Atlantic. Their complex population structure is poorly known and is mostly based on data from tag-recapture studies. The limited data available suggest that size, sex and reproductive stage influence the spatial and temporal distribution of blue sharks. Despite the declines in abundance in the North Atlantic, blue shark stock structure is still poorly understood. This genetic population study aims to understand the genetic stock structure of blue sharks within the Atlantic Ocean. The sampling scheme targeted young-of-the-year and small juvenile (<2yr) blue sharks, the most resident component of this species, in three known Atlantic nurseries (around the Azores, off Portugal and off South Africa). In addition, thirteen highly polymorphic nuclear microsatellite markers and a 426-bp fragment of the mtDNA Control Region were used to estimate the genetic diversity of the blue sharks. Significant genetic heterogeneity was found among blue shark nurseries from the Atlantic Ocean, suggesting that the northern nurseries (off Portugal and Azores) have distinct haplotype frequencies compared to the southern nursery (South Africa). Moreover, Factorial Component Analysis, based on individual multilocus genotypes, further suggests structuring within the North Atlantic nurseries (Azores vs Portugal). Data analysis also indicated a putative signal of temporal genetic structure among blue shark cohorts, both within and among nurseries. These results are discussed in terms of their implications for current conservation measures for blue shark populations in the Atlantic.

Keywords: *Prionace glauca,* blue shark, genetic population structure, cosmopolitan distribution, nurseries.

Resumo

O tubarão azul (*Prionace glauca*), um dos tubarões carcarinídeos mais abundantes, é uma espécie oceânica e epipelágica com distribuição mundial. Este extraordinário migrador pode percorrer distâncias longas, que podem chegar a 7176 km, dentro de bacias oceânicas. No entanto, a sua estrutura populacional complexa é ainda pouco conhecida, e essencialmente baseada em dados provenientes de estudos de marcação e recaptura. Os dados disponíveis sugerem que o tamanho, o sexo e o estado de maturação sexual influenciam a distribuição espacial e temporal do tubarão azul. O tubarão azul é também a principal espécie acessória das pescarias de espadins e atuns no Oceano Atlântico, e já registou declínios na sua abundância no Atlântico Norte. Este estudo de genética populacional tem como objectivo a compreensão da estrutura genética de stocks de tubarões azuis no Oceano Atlântico. O desenho experimental incidiu apenas em amostras de tubarões azuis recém-nascidos ou juvenis pequenos (<2anos), correspondendo à componente mais residente da espécie, em três maternidades ("nurseries") registadas para o Oceano Atlântico (ao largo dos Açores, Portugal e África do Sul). Dois tipos de marcadores moleculares, i.e. treze microsatellites nucleares extremamente polimórficos e um fragmento de 426-pb da região de controlo do ADN mitocondrial, foram utilizados para estimar a diversidade genética das amostras de tubarões. Foi detectada heterogeneidade genética significativa entre maternidades de tubarão azul do Oceano Atlântico, sugerindo a distinção entre nurseries a Norte (Portugal e Açores) e a Sul (África do Sul) com base em diferenças nas frequências haplotípicas. Para além disso, a Análise Factorial de Componentes das composições genotípicas multilocus entre indivíduos também sugeriu a estruturação entre nurseries do Atlântico Norte. Finalmente, a análise dos dados sugeriu ainda um sinal de estrutura genética temporal entre cohorts de tubarão azul, dentro de cada nursery mas também entre nurseries. Os resultados obtidos são discutidos tendo em conta as implicações nas actuais medidas de conservação das populações de tubarão azul do Atlântico.

Palavras-chave: *Prionace glauca*, tintureira, estrutura genética populacional, distribuição cosmopolita, maternidades.

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List of Acronyms

AMOVA – Analysis of Molecular Variance

DNA – Deoxyribonucleic acid

EU – European Union

FCA – Factorial Correspondence Analysis

FL – Fork Length

gDNA – genomic DNA

HWE – Hardy-Weinberg Equilibrium

ICCAT – International Commission for the Conservation of Atlantic Tunas

IUCN – International Union for the Conservation of Nature

MPA – Marine Protected Areas

MSY – Maximum Sustainable Yield

mtDNA – Mitochondrial DNA

mtDNA CR – Mitochondrial DNA Control Region

PCA – Principal Component Analysis

PCR – Polymerase Chain Reaction

YOY – Young-of-the-Year

Introduction

The Requiem sharks (family Carcharhinidae) comprise the largest family of shark-like Elasmobranchs and are one of the most important groups of sharks in terms of abundance and biomass in the world (Compagno, 1984). The name Carcharhinidae is derived from the greek words *karcharos* and *rhinos*, that etymologically mean sharks with sharp noses. The generally distinguishing morphological features of these sharks are: medium to large body sizes (e.g. 85 -251 cm fork length), head not anteriorly expanded, round eyes with nictitating eyelids, absence of nasoral grooves or barbels, general absence of spiracles, precaudal pits present, two dorsal fins, one anal fin, and different teeth morphology in upper and lower jaws (Compagno, 1984). The most popular and charismatic sharks, like the tiger and bull sharks, are part of about 50 species of this family (Compagno et al., 2005).

The Blue Shark *Prionace glauca*

One of the most abundant carcharhinid sharks is the blue shark *Prionace glauca* (Linnaeus, 1758). Blue sharks are large vagile sharks growing up to 334 cm fork length (FL; Megalofonou et al., 2009) and are morphologically characterized by the following diagnostic features: a long, narrow body with a slender snout, large eyes, papillose gillrakers on internal gill arches, absence of spiracles, weak lateral keels on the caudal peduncle, and position of dorsal fin closer to the pelvic than to the pectoral fins (Compagno, 1984; Nakano & Seki, 2003).

The blue shark body is built for fast and continuous swimming with little effort, allowing the species to fill niches swept by strong currents like seamounts (Sims, 2010). The highly hydrodynamic blue shark body morphology is expressed in several morphological features: the torpedo-like body shape, similar to a long glider with slender fuselage, allows for fast and strong swimming while the long, wing-like pectoral fins allow them to ride on currents and gyres, functioning also as a strong vertical stabilizer (Nakano & Seki, 2003; http://elasmo-research.org/, accessed, 11-03-13). This body shape enables blue sharks to make long migrations as well as fast hard turns during predation, or even to get away from other fast swimming fish.

A specific adaptation of blue sharks to the open ocean habitat is the countershading body coloration, ranging from a deep blue on the dorsal side to a bright white on the ventral side (Nakano & Seki, 2003). It is also seen in many other oceanic pelagic fish, like tuna or swordfish (Karleskint et al., 2009). This adaptation to the pelagic environment is a camouflage: the dark back matches the dark surrounding water if seen from above, and the white belly matches the bright lightened surface if seen from below (Karleskint et al., 2009).

Geographic Distribution

As one of the widest ranging species of the family Carcharhinidae, blue sharks are found in the Indian, Pacific and Atlantic oceans at latitudes between 60ºN and 50ºS (Stevens et al, 2010). Oceanic, epipelagic and littoral environments in tropical, subtropical, and warm-temperate seas are their habitat (Kohler et al., 2002; Skomal & Natanson 2002; Kleiber et al., 2009). Within the Atlantic Ocean, *P. glauca* is known to occur in the west from Newfoundland to Argentina, and in the east from Norway to South Africa, including the Mediterranean Sea and the Gulf of Mexico (Kohler et al., 2002; Clark et al, 2006; Megalofonou et al., 2009). This species is also vertically distributed between the surface and 1160 m depth (Kohler et al., 2002; Litvinov, 2006; Queiroz et al., 2010).

Diet

Blue sharks generally feed at night, and the main preys of this apex predator are cephalopods and pelagic teleost species locally abundant, and some demersal fish (Henderson et al., 2001; Kim et al., 2011; Preti et al., 2012). Despite the high selectivity of prey items found in the blue shark diet, they are also known to be opportunistic feeders (Henderson et al., 2001). Seabirds, crustaceans, marine mammals and other elasmobranchs caught on nets are occasional prey items without an important role on the diet of blue sharks (Kim et al., 2011; Preti et al., 2012).

Blue sharks have developed many morphological adaptations for feeding in the open ocean environment, where food may be scarce and patchily distributed. For instance, as other oceanic sharks, they developed visual systems highly adapted to

spot mobile and active prey (http://elasmo-research.org, accessed, 11-03-13; Jordan et al., 2013).

Reproductive Cycle

Blue sharks are placental viviparous species, i.e. the development of the embryos occurs inside the maternal uteri, with a gestation period of 9 to 12 months (Pratt, 1979; Castro & Mejuto, 1995). Parturition occurs during the Spring and Summer in the Atlantic Ocean, and is immediately followed by mating (Pratt, 1979). *P. glauca* is a relatively fecund and fast-growing elasmobranch species. In the North Atlantic, the females produce an average litter size of 36 pups (Nakano & Seki, 2003; Sousa, 2009), with neonate blue sharks measuring 30-43 cm FL on average (Nakano & Stevens, 2008).

Male blue sharks reach sexual maturity with an average size of 183 cm FL and an average age of 6 years (Pratt, 1979; Nakano & Stevens, 2008). However, female blue sharks undergo a sub-adult phase of two years (145-185 cm FL), during which females are ready for copulation but are still developing the sexual organs needed for gestation (Nakano & Stevens, 2008). Female blue sharks reach full sexual maturity at an average size of 185 cm FL and an average age of 4-5 years, which is when the diameter of ovarian eggs is largest (>1.0 cm; Pratt, 1979; Nakano & Stevens, 2008).

Population Structure

The blue shark exhibits a complex population structure as showed by tag-recapture studies (Kohler *et al.*, 1998; Fitzmaurice *et al.*, 2005; Mejuto *et al*, 2005; ICCAT, 2009; Green et al., 2009). Like many elasmobranchs, *P. glauca* exhibits spatial segregation by size, sex and reproductive stage (Simpfendorfer et al., 2002; Nakano & Seki, 2003; Robbins, 2007). Segregation by size occurs according to latitude (Nakano & Seki, 2003): there is an increase in the length of blue sharks from temperate and sub-arctic latitudes towards the tropical belt (Kohler et al., 2002; Nakano & Seki, 2003; Carvalho et al., 2011; Voghler et al., 2012). On the other hand, the pattern behind segregation by sex in *P. glauca* is thought to be due to separate food niches for the two sexes as suggested by different teeth morphologies between males and females (Compagno, 1984; Nakano & Seki, 2003; Litvinov, 2006).

Segregation by reproductive stage is intimately related to segregation by sex (Kohler et al., 2002; Mejuto & García-Cortèz, 2005; Litvinov, 2006; Tavares et al., 2012). Blue sharks smaller than 60 cm FL already exhibit sexual segregation (Litvinov, 2006) although the young remain in confined areas usually near the coast, i.e. nurseries or kindergardens, until reaching 109 cm FL (Stevens, 1990; Nakano & Seki, 2003; Litvinov, 2006). Within nurseries, schools of females or of males are formed due to distinct feeding sources (Litvinov, 2006). These nurseries seem to be located in areas of high prey biomass, thus maximizing juvenile survival and growth, or where juveniles find protection from predators (Simpfendorfer & Milward, 1993; Nakano & Stevens, 2008; Francis, 2013). In the Atlantic Ocean, the locations reported as having high incidence of juvenile blue sharks (<150 cm FL) are located off mainland Portugal, off the Azores and off western South Africa (Kohler et al., 2002; Silva et al. 2010; Queiroz et al., 2012). The Mediterranean is also considered as a mating area (Hemida & Capapé, 2003) and as a nursery ground, particularly the Adriatic Sea (Megalofonou et al. 2009).

Male aggregations, or male clubs, have been found in the vicinity of nursery areas (Litvinov, 2006). Juvenile female blue sharks with 100-180 cm FL migrate to oceanic regions where male clubs are formed and mating takes place (Simpfendorfer et al., 2002; Nakano & Stevens, 2008). After copulation, the females usually move out from the male clubs though some pregnant females may remain there until close to parturition (Litvinov, 2006). Parturition is thought to take place closer to shore (Tavares et al., 2012; Vögler et al., 2012).

Movements and Migrations

Blue sharks are excellent travelers that can migrate between northern and southern hemispheres as well as between eastern and western margins of ocean basins (Stevens, 1990). They often make transoceanic movements and the maximum recorded distance traveled by an individual was 7176 km (Stevens, 1990; Kohler et al., 2002; ICAAT, 2009; Queiroz et al., 2010; Costa et al., 2012). Despite their high dispersal ability, *P. glauca* exhibits site fidelity to coastal or pelagic oceanic locations like seamounts, continental shelves, canyons or oceanographic fronts (Bigelow *et* al., 1999; Litvinov, 2006; Morato et al., 2010; Jolly et al. 2011; Queiroz et al., 2012), which harbor high nutrient concentrations supplied by runoff and/or thermal front boundaries leading to higher primary productivity (Queiroz et al., 2012). These regions are an

exception in a generally oligotrophic environment (Hazen et al., 2013). However, these oases of the open ocean play an essential role as feeding, mating and parturition grounds for blue sharks (Litvinov, 2006; Queiroz et al., 2012).

Transatlantic movements were reported along the North Atlantic gyre, suggesting exchange of sharks between the eastern and western margins (Stevens, 1990; Silva et al, 2010). Males may move in and out of male clubs but tend to occur in the western North Atlantic during the summer to take advantage of the locally abundant food resources (Simpfendorfer et al., 2002). During winter, males stay east of the Gulf Stream (Kohler & Turner, 2008). With the arrival of sub-adult females, mating occurs at latitudes of 20-30ºN between May and June (Pratt, 1979; Nakano & Seki, 2003; Kohler & Turner, 2008). With higher water temperatures during the Spring, the sharks move northward to Newfoundland in April and May (Kohler & Turner, 2008).

Following copulation, sub-adult females in the western North Atlantic margin move eastward following the Gulf Stream to the northeast Atlantic at the beginning of Summer and until Fall (Simpfendorfer et al., 2002; Kohler & Turner, 2008). During the Winter, many pregnant females tend to concentrate in tropical waters between the African coast, and Madeira and Canary islands (Steven, 1990; Castro & Mejuto, 1995; Kohler & Turner, 2008; Tavares et al., 2012). It is possible that they stay there until egg fertilization and attaining sexual maturation in the following Spring (Stevens, 1990; Kohler & Turner, 2008). After parturition, juvenile sharks stay on a confined area (i.e. nursery) and do not migrate (Kohler & Turner, 2008).

Adult shark movements also revealed patterns of north-south migrations in the North Atlantic (Nakano & Seki, 2003). For instance, large adult females (185 cm FL) move to the coast of England in early Summer and are posteriorly followed by smaller males (Stevens, 1990; Nakano & Seki, 2003). It is also during the Summer that other northward movements to off Ireland, Scotland, North Sea, Baltic Sea and Norway, have been recorded for blue sharks (Aasen, 1966; Kohler & Turner, 2008). Some large adult females move South to off the Canary Islands and northern coast of Africa in the Spring and Summer, being posteriorly followed by smaller males during the Winter (Stevens, 1990).

Pelagic Fisheries

Blue sharks are the most frequent by-catch of swordfish and tuna longline pelagic fisheries, as well as of recreational fisheries worldwide (Castro & Mejuto, 1995;

Kohler et al., 1998; Nakano & Seki, 2003; Aires-da-Silva *et al*. 2008). This species had none or little commercial value during the 1950's and 1960's (Kohler et al., 1998), but an increased demand for blue shark by European markets in the 1990's led to increased landings of *P. glauca* by the Spanish pelagic longline fishery (Mejuto et al. 2002). Currently, this species is strongly targeted in Europe and used for human consumption like shark-fin soup, in fish meals, for leather or pharmaceutical purposes like eye drops (Nakano & Seki, 2003; Fordham, 2006; Aires-da-Silva *et al*. 2008).

Up to now, most stock assessment models consistently estimated that blue shark stocks in the Atlantic were not overfished (i.e. the population biomass is not below the Biomass of Maximum Sustainable Yield (MSY)), and that overfishing was not occurring (i.e. fishing mortality is below the level needed to maintain MSY) (ICCAT, 2009). However, the lack of complete and realistic historical records of catches and the assumptions of the assessment models render these results as uncertain (Babcock & Nakano, 2008). Indeed, at the regional scale, declines in catch-per-unit-effort of 30-60% were estimated for blue sharks off the Northwest Atlantic over the past 15 years (Baum et al., 2003; Aires Silva, 2008). Therefore and contrary to previous expectations for the North Atlantic, blue shark catches may be beyond the MSY (Cortés et al., 2010). Furthermore, the volume of blue shark fins marketed in the Asian fin market are three to four times the globally reported value of blue shark landings (Clarke et al. 2006; Aires-da-Silva & Galluci, 2007; Pikitch et al., 2008), showing that catches are grossly under-reported. This may be due to the discarding of shark carcasses at sea after finning (Tavares et al., 2012). The unreported catches of sharks can mask the potential for current overfishing in a variety of species that is not evident in official catch statistics. Moreover the slow growth and long population doubling times of these species makes them susceptible targets to overfishing (Castro & Mejuto, 1995; Rodrigues-Filho et al., 2012). Consequently, the top-down effect on trophic food webs can lead to an increase of mid-level consumers, shifts in species interactions, and trophic cascades (Ferretti et al., 2008; Markaida & Sosa-Nishizaki, 2010).

Given the high fishing pressure exerted on blue sharks worldwide, the International Union for the Conservation of Nature (IUCN) has recently changed their assessment of the blue shark status from Vulnerable to Near Threatened (Megalofonou et al., 2009; Stevens, 2009; Stevens et al., 2010). At the same time, ICCAT (International Commission for the Conservation of Atlantic Tunas) banned finning by its member nations in the Atlantic, because overexploitation may lead to irreversible consequences (Pikitch et al., 2008). Prior to 2003 the European Union, the biggest exporter of shark fins to Asia, prohibited shark finning by EU vessels (Council Regulation (EC) No. 1185/2003). However, the banning was compromised by "special

fishing permits" under article 4: finning was allowed if the vessels had the capacity to preserve fins and carcasses, and the two products could be landed separately at different harbors (EU, 2010). Spanish and Portuguese fishing vessels made ample use of this special fish permit (Shark Alliance, 2012), exhibiting the highest catches of blue sharks (Camhi *et al*., 2008). Nevertheless, in 2012, the European Parliament voted in favor of the landing of whole sharks with "naturally attached fins" by all member countries (A7-0295/2012), ending with the exceptions for countries like Spain and Portugal. Despite the EU finning ban, *P. glauca* fisheries are generally unregulated worldwide and with a tendency for increased fishing effort (IUCN, 2013).

Although the blue shark is among the most productive of pelagic sharks (Cortés, 2000), its productivity is lower when compared to teleost species (Miller & Kendal, 2009; Lowerre-Barbieri et al., 2011) and thus it is still highly susceptible to overfishing as a by-catch of fisheries targeting more productive teleost species like tunas and billfishes. It is also known that their population growth rates are strongly dependent on the survival of juvenile sharks up to four years old (Aires-da-Silva & Gallucci 2007; Petersen et al., 2009).

Stock Structure, Management and Conservation

The stock structure of *P. glauca* is still uncertain (Kohler et al., 2002; Nakano & Seki, 2003). However it is known that its distribution is dependent on seasonal variations of sea temperature, on reproductive stage and on the availability of prey (Kohler & Turner, 2008). Recently, ICCAT assumed that there are at least three stocks of blue shark in the Atlantic Ocean: one in the Mediterranean Sea, a second in the North Atlantic and a third in the South Atlantic (ICCAT, 2009). This stock structure was based on data from tag-recapture studies showing patterns of north-south movements in the South Atlantic (Hazin *et* al, 1990) as well as on both sides of the North Atlantic (Nakano & Seki, 2003; Queiroz et al., 2005; Kohler & Turner, 2008). Also, based on the low density of blue shark recaptures along the Equator, separate stock units are assumed for the North and the South Atlantic (Nakano & Seki, 2003; Litvinov, 2006). The North Atlantic stock has been proposed to exhibit a cyclical migration of blue sharks occurring in a clockwise direction between 30-50ºN (Kohler et al., 2002; Skomal & Natanson, 2002; Fitzmaurice et al., 2005). Tag-recapture studies also reported limited movement of blue sharks between the Atlantic and the Mediterranean Sea

leading to the assumption of a single Mediterranean stock separated from the two stocks of the Atlantic Ocean (Kohler & Turner, 2008; ICCAT, 2009).

The conservation and sustainable exploitation of fishery resources requires knowledge of the stock structure of the exploited species (Hueter & Simpfendorfer, 2008; Nakano & Stevens, 2008). Previous studies on *P. glauca* have focused on a variety of topics including its geographic range, extent of migrations, stock identity, movements, abundance, age and growth, mortality and behavior (Hazin et al., 1994; Lessa et al., 2004; Queiroz et al., 2005; Litvinov, 2006; ICCAT, 2009; Peterson et al., 2009; Queiroz et al., 2010). However knowledge on the genetic population structure of blue sharks is still lacking. The most common techniques used in previous studies of blue shark population structure were tag-recapture methods, or satellite telemetry tracking (Kohler et al., 1998; Weng et al., 2005). Yet, there are limitations of these methods in the study of population structure and dispersal patterns of elasmobranchs (Queiroz, 2010). For instance, in the case of satellite telemetry tags, the cost per tag limits the number of studied sharks while the large size of the tags limits the maturity stage (size) of the tagged sharks (Queiroz, 2010). Other constraints of these methods are the premature release of the tags, which decrease the time collecting data, and the assignment of the shark behavior to the correspondent function (Begg & Waldman, 1999; Queiroz, 2010). In the case of tag-recapture studies, the limitations are related with the lack of information registered between the tagging and the recapturing of the sharks. The movements of the fishes between those procedures may gather important evidence about the life history of the population or species of shark (ICCAT, 2013). Moreover recapture is dependent on the fishing effort of the fisheries, i.e. the recaptured sharks are usually from fishery locations and the reporting of recaptures is dependent on the volunteering of the fishermen. As so a bias is associated to the recaptures data that do not include sharks of other locations and to the knowledge of the movements of sharks (ICCAT, 2013). Additionally despite transoceanic movements being reported, long-distance movements do not necessarily imply gene flow among distant locations (Begg & Waldman, 1999; Heist, 2008).

Recently, genetic studies started complementing the above mentioned techniques in studies of Elasmobranch population structure, and particularly of blue sharks (Ovenden et al., 2009; Queiroz, 2010). Systematic and phylogeographic studies of most animals are often done using sequences of mitochondrial and nuclear gene regions (Avise, 1998; Padial et al., 2010). Nuclear microsatellites are highly polymorphic codominant markers, with a range of evolutionary rates that are best suited to study population-level questions (Sunnucks, 2000: Heist & Feldheim, 2004). The use of both mitochondrial DNA (mtDNA) and nuclear microsatellites markers can

be integrated to reveal distinct parts of each species' population structure and evolutionary history (Zhang & Hewitt, 2003).

However, there are still few population genetic surveys completed among cosmopolitan elasmobranch species (e.g. Quattro et al., 2005; Schultz et al., 2008; Portnoy et al., 2010), though conservation and management of commercially exploited species requires detailed information regarding the population structure of these taxa (Rodrigues-Filho et al., 2012; ICES, 2012).

Genetic Population Studies of Elasmobranchs

Genetic studies in Elasmobranchs have only been conducted since the early 1990s (Heist et al., 1995) and have focused mostly on carcharhinid sharks, and particularly on coastal pelagic species (Annex I). These studies revealed that the distribution patterns of intraspecific genetic variation within Elasmobranchs are strongly influenced by the species' dispersal ability (vagility), distribution range and life history strategy (e.g. Heist, 2008; Veríssimo et al., 2010).

Dispersal ability in elasmobranchs is intimately related with body size, type of habit and habitat (Musick et al., 2004). Large, pelagic and oceanic shark species present the greatest dispersal ability and widest geographical ranges (Musick et al. 2004; Heist, 2008). These species are often widely distributed, have weaker genetic divergence among populations and tend to exhibit low levels of genetic diversity, as in the shortfin mako *Isurus oxyrinchus* (Schrey & Heist, 2003) and the basking shark *Cetorhinus maximus* (Hoelzel et al., 2006). Contrarily, small, benthic and coastal elasmobranchs species have more limited dispersal ability and/or more restricted distribution ranges (Feldheim et al., 2001; Musick et al., 2004). These species often show stronger genetic differentiation among populations and at comparatively smaller geographic scales (e.g. the gummy shark *Mustelus antarcticus* Gardner & Ward, 1998; the thornback ray *Raja clavata* Chevolot *et al*., 2006).

The geographic distribution of genetic variation within elasmobranchs is also related with their life strategies (Heist, 2004). In many carcharhinid sharks, like the bull shark *Carcharhinus leucas,* the blacktip shark *C. limbatus*, the sandbar shark *C. plumbeus* and the lemon shark *Negaprion brevirostris*, philopatry of females to nursery areas lead to genetic divergence among juveniles at different nursery grounds and the existence of distinct genetic stocks/populations (Hueter et al., 2005; Keeney & Heist, 2006; DiBattista et al., 2008; Portnoy et al., 2010; Tillett et al., 2012; Mourier & Planes,

2012). Patterns that suggest female philopatry to nursery areas and male-mediated gene flow have also been noted in sharks of the Lamnidae family, like in the mako shark *Isurus oxyrinchus* and the white shark *Carcharodon carcharias*, which are characterized by widespread distributions, oceanic habit and highly migratory nature (Pardini et al., 2001; Schrey & Heist, 2003; Jorgensen et al., 2010).

Contrarily to most species of its family, the blue shark is a highly migratory oceanic carcharhinid shark with a worldwide distribution range. It has thus great potential for widespread gene flow among distant locations and for genetic homogeneity among very distant locations, making the detection of genetic population structure more challenging. Yet, cosmopolitan marine species may still reveal deep genetic divergences among populations (Quattro et al., 2005). For instance, internal fertilization, viviparity and reproductive philopatry can contribute to strong genetic population structure in widely distributed species (Rodrigues-Filho et al., 2012; Muths et al., 2013).

A previous attempt to elucidate the genetic population structure of blue sharks found no significant population genetic structure within the North Atlantic (Queiroz, 2010). The absence of genetic structure supported panmixia within the sampling area and suggested a single stock management unit for the North Atlantic blue sharks (Queiroz et al., 2010). However, these results may be a consequence of the opportunistic sampling of adult sharks followed in the previous study. Specifically, highly migratory adults belonging to distinct population units may co-occur at a given feeding area for a period of the year. The comparison of sample collections comprised of individuals of mixed origin, i.e. from different population units, may result in no detection of genetic divergence among them.

In order to adequately assess the genetic diversity within any given species and to test for genetic divergence among its populations, it is important to choose the right genetic marker(s) and to design the correct sampling strategy. Heist (2008) highlights the importance of locating nurseries as a source of population recruits and to infer the stock structure of pelagic sharks, since nurseries harbor the non-migrant component of the populations (i.e. neonates and small juvenile sharks) and, consequently, allow some degree of isolation among population units. Neonates near nurseries may be the best target to find genetically distinct stocks (Keeney et al., 2003; Heist, 2008).

Objectives

The aim of the present analysis is to test whether there is significant genetic differentiation among blue shark nurseries in the Atlantic Ocean. To test this hypothesis, highly polymorphic molecular markers, namely nuclear microsatellite loci and nucleotide sequences from the mitochondrial DNA control region, will be used to estimate the levels of genetic diversity and genetic differentiation among sample collections from reported nursery grounds in the Atlantic Ocean. In order to exclude a confounding signal from migratory adult sharks, this study focus on young-of-the-year (YOY) and small juvenile blue sharks (<130 cm FL) collected in the reported Atlantic nursery areas, namely off Portugal, Azores and South Africa.

Material and Methods

Sampling

Sampling locations correspond to three known *P. glauca* nursery areas within the Atlantic: off the Azores, off mainland Portugal and off western South Africa (Fig. 1). The nursery area off mainland Portugal is herein designated as Portugal nursery. A total of 147 individual blue sharks were sampled from commercial and recreational fisheries operating in the Atlantic Ocean between 2003 and 2008 (Table 1 and 2). Tissue samples were obtained from dorsal fins, muscle or heart tissue (~1cm^3) of juvenile blue sharks under 130 cm FL, preserved in 96% ethanol and stored at room temperature. The collected samples corresponded to young-of-the-year (YOY), 1- and 2-year old blue sharks, based on the age-at-length estimates of Skomal & Natanson (2002).

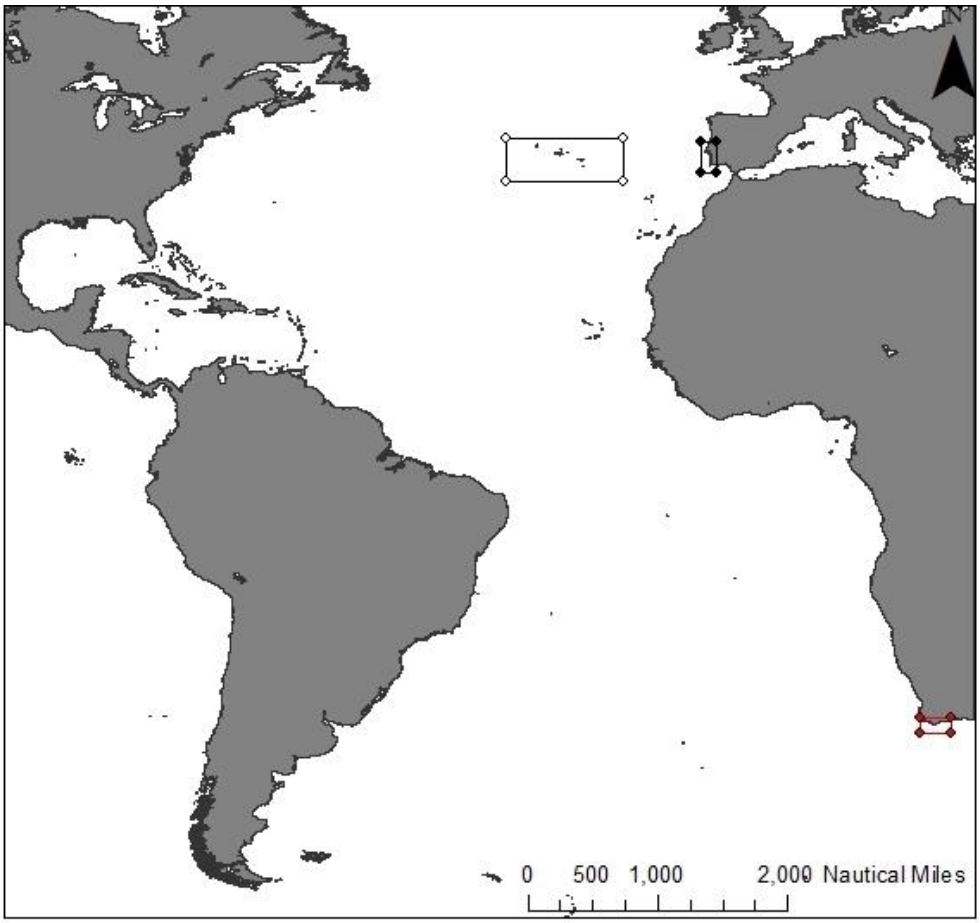

Figure 1. Sampling locations of Atlantic blue sharks *P. glauca* highlighted. The color codes represent the three nurseries: black-Portugal, white-Azores and red-South Africa.

Table 1. *P. glauca* collected from Atlantic nurseries. Total number of sampled individuals (N), sex ratio, range of fork length (FL), and cohorts are presented for each nursery.

Local	N	Sex-ratio	FL (cm)	Cohorts
Portugal	58	1:0.51	65-120	2002-2008
Azores	52	1:2.18	88-120	2004-2006
SouthAfrica	37	1:1.25	85-126	2006, 2007

Table 2. *P. glauca* collected from each Atlantic nursery and from each cohort.

		Nurseries			
		Portugal	**Azores**	**South Africa**	Total
Cohorts	**2002**	8			8
	2003	22			22
	2004	3	8		11
	2005	3	36		39
	2006	5	8	16	29
	2007	16		21	37
	2008	1			1
	Total	58	52	37	

Molecular Data Collection

Total genomic DNA (gDNA) was extracted from each tissue sample using the EasySpin® Genomic DNA Tissue Kit (Citomed, Lisbon), according to the manufacturers' instructions. The quality and quantity of the gDNA was checked through gel electrophoresis of 2 µl of extracted DNA volume in 0,8% agarose gels (w/v) stained with GelRed (0.175X) (Biotium, Inc., Hayward, CA, USA) and run at 200 V for 15 min in TBE 0,5X (Tris 89mM, Boric Acid 89mM, EDTA 2mM; pH 8.0). Each gel was visualized under ultra-violet light on a Biorad Universal Hood II Quantity One 4.4.0 enabling the relative quantification of DNA. Samples exhibiting strongly stained bands were diluted in ultra-pure water 1/3 to 1/7 in order to avoid inhibitors' influence during downstream polymerase chain reaction (PCR) amplification.

Nuclear Microsatellites

A set of 10 and 8 nuclear microsatellite loci developed specifically for *P. glauca* were obtained from Fitzpatrick et al. (2011) and Mendonça et al. (2012), respectively (Table 3). The DNA amplification of each primer pair via PCR was optimized using two individual samples, and by conducting a temperature gradient for the annealing temperature of ± 5 ºC around the respective published optimum annealing temperature. Minor adjustments were also made to the number of PCR cycles (30-35) to obtain optimum PCR amplification yields. The presence and quality of amplicons were checked through gel electrophoresis as mentioned above, but on 2% agarose gels (w/v). A size ladder (*Marker 5*, Eurogentec) was run in each gel to confirm the amplification of the expected fragment length.

Out of initial 18 loci, loci Pgla09, TB05, TB07 and TB08 did not amplify successfully. The 14 loci for which successful and consistent PCR amplification was obtained, were combined into two multiplex reactions of 8 loci and 6 loci each where the forward primer of each locus was labeled with a fluorescent tail of one of four dyes (6-FAM, VIC, NED or PET; Applied Biosystem). These multiplex reactions were further optimized for primer concentration (primer F: 0.08-0.4µM; primer R: 00.8-4 µM) and for the duration of each temperature step (e.g. 30 to 90 sec), through PCR amplification of 4 samples plus a negative control. Presence and quality of PCR amplification of each multiplex reaction were checked by gel electrophoresis in 2% agarose gels (w/v), and

visualized in a Biorad Universal Hood II Quantity One 4.4.0 as mentioned above. Again, a size ladder (*Marker 5*, Eurogentec) was added to the gels to confirm the amplification of the expected fragment length. Considering the yield of the PCR amplification, the amplicons were diluted up to 1/5 and run on an ABI 3130xl Genetic Analyzer (AB Applied Biossystems). One of the multiplex reactions was successfully optimized for a total of 6 loci, while the other was divided into a smaller multiplex reaction with 4 loci plus 4 additional uniplex (single locus) PCR reactions (Table 3, Annex II).

Each blue shark sample was individually genotyped for the 14 nuclear microsatellite loci selected out of the initial set of 18 loci. Each microsatellite locus or set of microsatellite loci was amplified via PCR in a 10 µl reaction volume. Each multiplex PCR reaction contained 5 µl of MM (*Taq* PCR Master Mix from Qiagen), 3 µl of ultra-pure water, 1 µl of primer mix (see Table 3 and Annex II for primer mix details) and approximately 1 µl of gDNA. Each uniplex PCR reaction contained 5 µl of MM (*Taq* PCR Master Mix from Quiagen), 3.2 µl of ultra-pure water, 0.04 µM of the T3 tailed primer F and 0.4 µM primer R, 0.4 µM of fluorescent dye and 1 µl of gDNA. All PCR reactions began with a hot start at 95ºC for 15min followed by: A) 17 cycles of a denaturation step of 95ºC for 30 sec, a touchdown annealing with a 0.5ºC decrease every cycle between 62-54ºC during 1.5 min, and an extension step at 72ºC during 30 sec; B) 15 cycles of a denaturation step as above, annealing at 54ºC during 45 sec and extension as above, and finally by C) 8 cycles of a denaturation step as above, annealing at 53ºC during 30 sec, and extension as above. A final extension step was conducted at 60ºC during 30 min. A few changes were done in the length of some steps for the uniplex PCR reactions: annealing was decreased by 15 sec at the 54ºC and 53ºC cycles of the PCR program. Fluorescently labeled PCR products were electrophoresed on 2% agarose gels (w/v) and visualized in a Biorad Universal Hood II Quantity One 4.4.0 as described above. Presence, length and quality of each marker were verified on the gel and diluted 1/1.2 if needed based on band brightness to avoid an excess of signal during electrophoresis on the ABI 3130xl Genetic Analyzer (ABI Applied Biossystems). One microliter of each diluted PCR product was added to 10 µl of deionized formamide and 0.2 µl of internal size standard (Genescan-500 LIZ, ABI) prior to each run. Genemapper software 4.1. (Applied Biosystems) was used to manually score the individual genotypes. After scoring, the locus Pgla-06 was excluded from the analysis due to problems of stuttering and allele calling.

Table 3. Summary details of analyzed *P. glauca* microsatellites **Locus:** marker name, **RM:** repeat motif, **T**: fluorescence tail, **PS:** forward primer sequence, **SR**: size range of PCR product (base pairs), **Tm**: temperature of annealing and reactions: **I** multiplex I, **II** multiplex II, **U** uniplex and **E** excluded.

Locus	RM	T	PS	SR (bp)	Tm (ºC)	Mix
Pgla01	(TCC)7-(TCC)3 TCG(TCC)5	NED	5' TTGATCTCGTCCATCTCCTTGTAG 3'	195-210	62	U
Pgla02	(TCC)5TCG(TCC)2 (TCG)2	NED	5' ACCCGACTCGCCAGGATTCACT 3'	124-148	60	U
Pgla03	(GGA)3AAA(GGA)4TGA(GGA)2	6-FAM	5' TATGGTGGTGTGCACAAGCAAGAG 3'	180-192	60	II
Pgla04	(TCT)4(TCC)6	6-FAM	5' TGCCTCCAGAGGCCTTGGACG 3'	215-224	62	II
Pgla05	(GT)27(GA)19	6-FAM	5' CAGATTCCTGTGTGGAGCACA 3'	186-260	60	I
Pgla06	(CA)2(GA)10CA	VIC	5' CTTTCGATGGTCTTTTGATGG 3'	117-141	60	U
Pgla07	(TCC)14	VIC	5' CAGGCCCTAGTGACCAAAGT 3'	199-232	60	I
Pgla08	(TCC)7(TCC)5	PET	5' CCTTCAACTTCCGGCTGGTGTT 3'	179-206	62	II
Pgla09	(GGA)6(GAA)5	-	5´AGCCGCTCACTCACTCTGC 3´	133-148	-	E
Pgla10	(GA)2CA(GA)7 (GACA)3CAGACA (GA)13	6-FAM	5´GGGACTGTGAGGCAGCAG 3´	139-159	54	U
TB01	(GA)9	6-FAM	5' TTGGTTGGGAAGAAGACTA 3'	150-162	55	II
TB02	(GT)14	VIC	5' TGCATTAAATCACCACAACTC 3'	218-247	55	II
TB04	(CT)8	6-FAM	5' ATGATCAAACTTAAACTGGGTGTC 3'	131-138	55	I
TB05	(CA)7	-	5´ GGCAGCCGTTTTCATCAAG 3´	167-198	-	E
TB07	(GT)5(GA)5	-	5' AGATAAATGGAGAGCTCACGACTG 3'	170-213	-	E
TB08	(TG)16AGGAA(GA)2CA(GA)5	-	5' TGTTGGATAGCAGCAGAC 3'	191-202	-	E
TB13	(TG)6	VIC	5' ATGGGGTCAGAAGCAGAGAA 3'	155-179	60	II
TB15	(CA)5TACG(CA)4	6-FAM	5' ATTTTAGGATGGCAGGTG 3'	310-319	60	I

Mitochondrial DNA Control Region

The mtDNA control region (CR) was amplified via PCR using the primers developed for the white shark *Carcharodon carcharias* by Pardini et al. (2001). The 5´end was amplified with the forward primer GWS F6 5´ TTGGCTCCCAAAGCCAAGATT 3´ and the 3´end was amplified with the reverse primer PheCacaH2
5´ CTACTTAGCATCTTCAGTGCC 3´ (Pardini et al., 2001). PCR amplification conditions, including the annealing temperature of the primers, was optimized using two individual samples and by testing an annealing temperature gradient of 45-55ºC.

The mtDNA CR fragment was amplified on a total of 122 individual blue sharks via PCR in 10 µl final volume reactions including 5 µl of MM (*Taq* PCR Master Mix (Qiagen), 3.2 µl of ultra-pure water, 0.4 µl of each primer (10 µM) plus 1 µl of gDNA. The PCR reactions started with a hot start at 95ºC for 5 min followed by 40 cycles of denaturation at 95ºC during 1 min, annealing at 65ºC during 45 sec, and extension at 72ºC during 1 min. A final extension step was performed at 72ºC during 10 min. PCR products and a negative control were checked by gel electrophoresis on 2% agarose gels as described above. All mtDNA CR amplicons were purified with ExoSap (USB Corporation, OH, USA) following the manufacturer´s guidelines, to remove the excess of primers and other reagents. The final sequencing reaction was done separately for the forward and reverse strands, using the BigDye cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The resultant amplicons were cleaned with Sephadex and run on an ABI 3130xl Genetic Analyzer (AB Applied

Biosystems). Quality and accuracy of nucleotide base assignment, manual edition of chromatograms and sequence alignment was performed in Geneious 6.1.2. (Biomatters Ltd). Sequence alignment was performed with the Geneious Aligner algorithm under default conditions, and confirmed by eye.

Data Analysis

For the microsatellite dataset, MStoolkit in Excel was used to create input files for other programs (e.g. Genepop, Fstat, Genetix, etc), to check for typing and genotyping errors, invalid alleles, and to verify if the samples were related or double sampled. Micro-Checker version 2.2.3 (van Oosterhout et al., 2004) was used to evaluate possible stuttering, allele dropout or null alleles. Genepop version 4.2 (Raymond & Rousset 1995; Rousset 2008) was used to confirm that the genotypic distributions of each locus within each population were in accordance with the Hardy-Weinberg Equilibrium (HWE) expectations. Deviations from HWE were also assessed for each locus within each population by calculating the inbreeding coefficient F_{IS} with FSTAT version 2.9.3.2 (Goudet, 2002). GenePop was also used to test for linkage disequilibrium between each pair of loci in each population.

Additionally, GeneAlex version 6.5. (Peakall & Smouse, 2012) was used to estimate the following measures of genetic variation: observed (H_O) and expected heterozigosities (H_E), and allele frequencies per locus. FSTAT version 2.9.3.2 was used to calculate the mean number of alleles and the allelic richness (R_S) index per sample collection. The latter index estimates allelic diversity standardized by sample size in order to account for differences in the number of sampled individuals among collections. Relatedness among individuals was accessed with GeneAlex to check the possibility of having more sibling or related individuals than expected by random mating within the same nursery or cohort.

Mitochondrial DNA control region diversity indices, namely number of polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity and average number of nucleotide differences between haplotypes were calculated in DnaSP version 5 (Librado & Rozas, 2009).

In order to visualize the genetic diversity at the nuclear microsatellite loci, a factorial correspondence analysis (FCA) based on the multilocus microsatellite allele composition of the individuals was performed using Genetix version 4.05 (Belkhir et al., 2004) for a) the three nurseries, b) all cohorts sampled across nurseries (with >10 individuals each), and c) all cohorts (with >10 individuals each) sampled per nursery. In

the FCA, the location of individual genotypes in the n-dimensional space is based on their allelic composition at the microsatellite loci, such that individuals with similar allelic compositions across loci are closely located while individuals with more distinct allelic compositions are plotted further apart. Total inertia and each axis' inertia were tested for significance by 10 000 randomizations with PCAGEN version 1.2. (Goudet, http://www2.unil.ch/popgen/softwares/pcagen.htm).

Levels of among-population genetic differentiation were calculated by means of pairwise F_{ST} for microsatellite genotype data and for mtDNA sequence data, using Arlequin version 3.5.1.3. (Excoffier & Lischer, 2010). Statistical power of pairwise F_{ST} tests (i.e. the probability of rejecting the null hypothesis of genetic homogeneity when it is false) and the corresponding alpha level (i.e. the probability of rejecting the null hypothesis of genetic homogeneity when it is true) were assessed using the POWSIM software (Ryman & Palm, 2006). Pairwise F_{ST} among nurseries was tested by running POWSIM simulations with a sampling scheme composed of 2 populations of 50 individuals each and 1 population of 40 individuals, and using the overall allelic frequencies observed at the 13 microsatellite loci and at 12 microsatellite loci excluding Pgla03. Each simulation was run with 10000 burn ins, 100 batches and 1000 iterations per batch.

Arlequin was also used to infer the hierarchical population structure within Atlantic blue sharks through an analysis of molecular variance (AMOVA). Three scenarios of genetic population structure were tested with the AMOVA: the first included all three Atlantic nurseries in the same group and tested for genetic homogeneity among nurseries; the second and third AMOVAs included two distinct groups and tested for genetic homogeneity between the South Atlantic (i.e. South Africa) vs. North Atlantic nurseries (i.e. Portugal and Azores), and between oceanic (Azores) vs. continental nurseries (South Africa and Portugal), respectively. The AMOVA analyses were based on allele frequency data in the case of the nuclear microsatellite data, and based on haplotype frequencies for the mtDNA CR sequence data.

Mitochondrial DNA CR haplotype networks (Polzin & Daneschmand, 2003) were constructed with the software Network version 4.6.0.0 (http://www.fluxus-engineering.com), using the maximum-parsimony approach (Polzin & Daneschmand 2003) and the median joining algorithm (Bandelt et al. 1999), with default parameters and a transition to transversion ratio of 9:4 (estimated from the data using DnaSP).

Results

Genetic Diversity of Atlantic Blue Shark Nurseries

A total of 147 individual blue sharks were screened for variation at thirteen nuclear microsatellite loci. Out of the 147, 140 individuals were completely genotyped, while 7 individuals miss data at one microsatellite locus each. Null alleles were detected by MicroChecker at the Pgla-02 and Pgla-07 loci for the Portuguese nursery, at the Pgla-10 locus for the Azorean nursery, and at the Pgla-03 and Pgla-07 loci for the South African nursery, due to an excess of homozygotes. However, all genotypic distributions were in accordance with HWE expectations for all locus/population combinations after Bonferroni correction (mean F_{IS} varied between -0.010 and 0.061; Table 4), except locus Pgla-03 for the South African sample (F_{IS} = 0.363, *P*-value = 0.001). The loci were not in linkage disequilibrium. The number of alleles per locus varied between 3 (Pgla-04 and TB15) and 37 (Pgla-05) (mean: 8.6) and the mean allelic richness (R_S) varied between 8 (South Africa) and 8.4 (Portugal) (Tables 4). Mean heterozygosities and R_S were similar among the sampled nurseries (H_O: 0.61-0.65; H_E: 0.62-0.66; R_S: 8-8.4). South Africa had fewer private alleles (6) while Portugal presented the highest number of private alleles (13) (Table 4).

A 426-bp fragment of the mtDNA CR was sequenced for 135 blue sharks, which resulted in 22 haplotypes. The haplotypes differed by 13 substitutions (at 12 polymorphic sites), of which 1 was an indel, 9 were transitions and 4 were transversions. Nine polymorphic sites were parsimoniously informative and 3 were singleton mutations. The mean number ± standard deviation of differences between different haplotypes was 3.56±1.88. Nucleotide diversity among all individuals was 0.006 and the corresponding haplotype diversity was 0.87.

Nine haplotypes were singleton haplotypes (i.e. occurred only in 1 individual in total). Haplotypes with 1 individual occurred in 42-57% while 33-42% occurred in >3 individuals in each nursery. Almost half of the haplotypes (i.e. 10) were shared among two or more nurseries. The South African collection revealed higher haplotype diversity (*h:* 0.89) while Azores revealed higher divergence between haplotypes (*k:* 2.65), when compared to Portugal (*h:* 0.84, *k:* 2.36) (Table 4).

Table 4 . Genetic diversity indices of the blue shark, *P. glauca* gathering microsatellite loci and mtDNA CR haplotypes for each sampled nursery: number of genotyped or sequenced samples (N), mean number of alleles (Mean A), mean allelic richness (Mean R_S), range of allelic richness per locus (R_S range), mean observed heterozigosity (H_O), mean expected heterozigosity (H_E), fixation indices (F_{IS}) and Private Alleles; number of haplotypes (H), haplotype diversity (*h*), nucleotide diversity (π), the mean number of nucleotide differences between haplotypes (*k*) and private haplotypes (Private H).

	Nuclear Microsatellites								Mitochondrial CR sequences					
	N	Mean *A*	Mean Rs	Rs range	H_O	H_E	F_{IS}	Private Alleles	N	H	*h*	π	*k*	Private H
Portugal	58	9.2	8.4	2.6-26.2	0.61	0.65	0.061	13	48	14	0.85	0.0060	2.57	6
Azores	52	8.8	8.3	2-26.3	0.64	0.62	-0.010	8	50	12	0.84	0.0062	2.65	3
SouthAfrica	37	8.0	8	3-23	0.65	0.66	0.024	6	37	12	0.89	0.0061	2.61	3

The haplotype network based on the mtDNA CR recovered several common haplotypes, all of which were shared by the three nurseries (haplotypes 1, 2, 4, 5 and 7). From those, other less frequent haplotypes were derived exhibiting more restricted distributions (e.g. haplotypes 11, 15 and 21). The reticulated appearance of the network is caused by similar haplotypes that have several possibilities of origin. Fluctuations of frequency in shared haplotypes among nurseries do not reveal a differential pattern of distribution. Out of 22 haplotypes 6 were shared by all Atlantic blue shark nurseries, while 2 were shared exclusively by Portugal and South Africa, and another 2 by Azores and South Africa. Portugal has 6 private haplotypes (43% of total haplotypes) while Azores and South Africa have only 3 private haplotypes (25% of total haplotypes), which are mostly singletons (Fig. 2; Annex III).

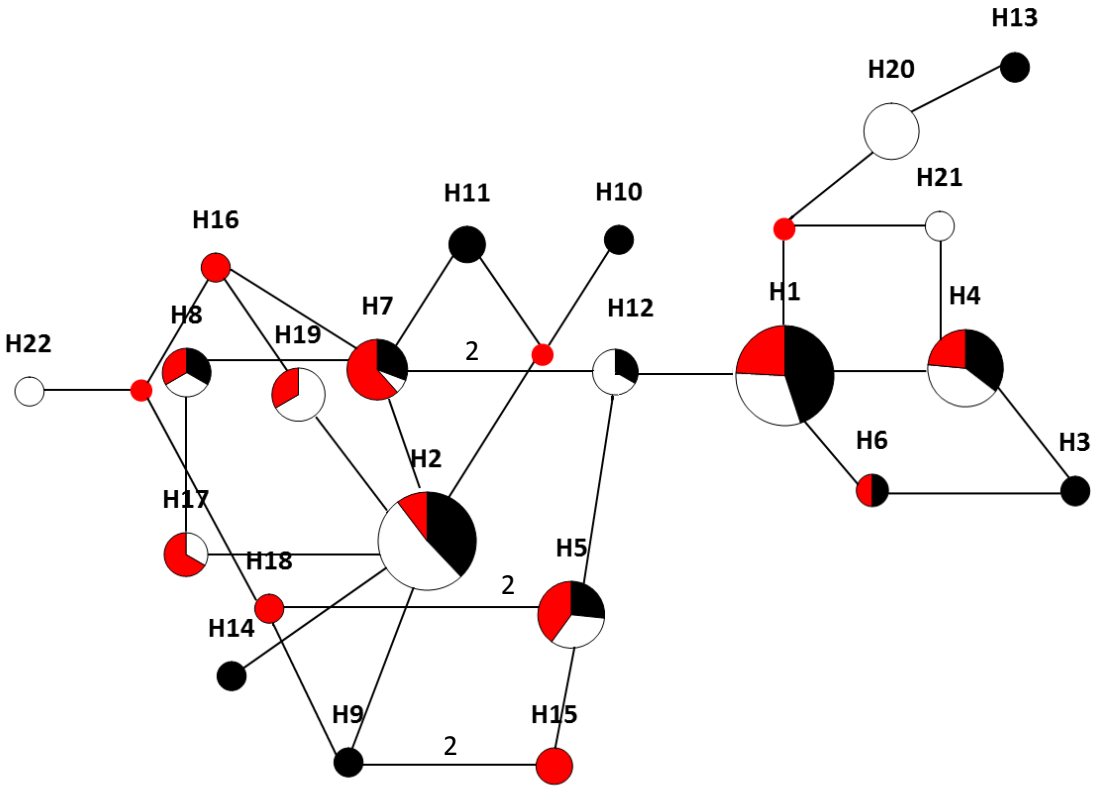

Figure 2. Maximum-parsimony haplotype network of the mitochondrial DNA CR region of the blue shark *P. glauca*. Size of circles are proportional to the absolute frequency in the total sample. The color codes represent the three nurseries: black-Portugal, white-Azores and red-South Africa, and the numbers above branches indicate the number of mutations between haplotypes if >1.

Pairwise relatedness analysis using nuclear microsatellite data revealed that within each nursery, the relatedness of individuals lies within the expected interval assuming random mating among individuals. Furthermore, the mean r index in each nursery is below or near 0, due to the low relatedness of the individuals (Fig. 3).

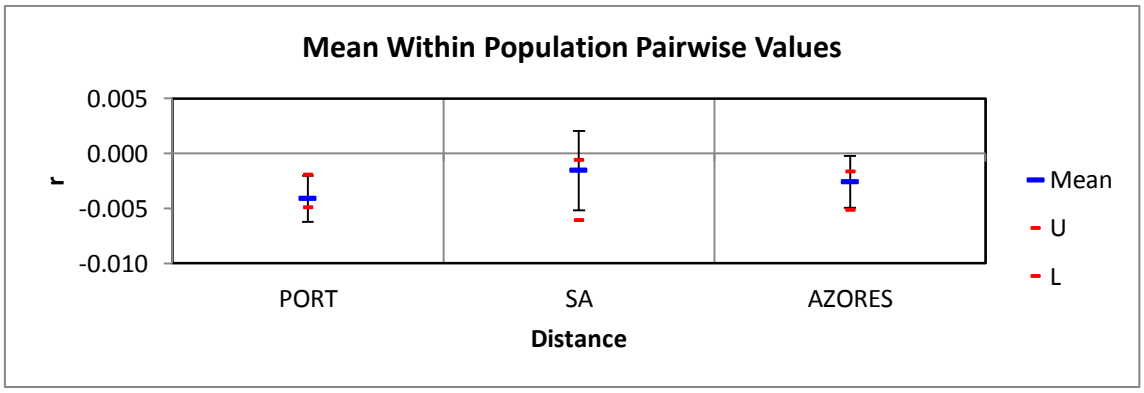

Figure 3. Pairwise relatedness within individuals of each Atlantic nursery of *P. glauca*.

Genetic Differentiation among Atlantic Blue Shark Nurseries

The FCA on the three Atlantic nurseries indicated that 100% of the inertia was explained by eigenvectors 1 and 2, and showed separate distributions of multilocus microsatellite genotypes among the sampled nurseries (Figure 4). This result suggests some level of genetic differentiation among Atlantic blue shark nurseries. PCA analysis was consistent with the FCA revealing a significant *P*-value for the axis 1 which separates southern (South Africa) from northern (Portugal and Azores) nurseries (PC1: F_{ST} = 0.0055, 70% of total inertia, *P* = 0.024; PC2: F_{ST} = 0.0024, 30% of total inertia, *P*= 0.975). Locus Pgla-03 presented deviations from HWE expectations and, therefore, two microsatellite datasets were used for further analysis: one with all 13 loci, and another with 12 loci excluding Pgla03 to rule out the possibility that HWE deviations could be affecting the results.

Pairwise F_{ST} values indicated low and non-significant genetic differentiation between Azores and Portugal ($F_{ST\ overall\ loci:}$ -0.0127, $F_{ST\ without\ Pgla03:}$ -0.0008, $F_{ST\ mtDNA\ CR:}$ 0.0067; *P*-value > 0.016), and between South Africa and Portugal ($F_{ST\ overall\ loci:}$ 0.0003, $F_{ST\ without\ Pgla03:}$ 0.0085, $F_{ST\ mtDNA\ CR:}$ 0.0085; *P*-value > 0.016). Nevertheless, low but significant genetic differentiation was found between the Azorean and South African nurseries ($F_{ST\ overall\ loci:}$ 0.007, $F_{ST\ without\ Pgla03:}$ 0.0082, $F_{ST\ mtDNA\ CR:}$ 0.0334; *P*-value < 0.016) (Table 5 and 6). Power analysis of pairwise F_{ST} tests using the current sampling design and nuclear microsatellite loci indicated that a pairwise F_{ST} level of 0.005 could be detected in 98% of the 10000 simulation runs, while the α value (type I error) was 0.036. The same analysis performed without the Pgla03 loci also detected a pairwise F_{ST} level of 0.005 in 98% of the 10000 simulation runs while the α value (type I error) was 0.032.

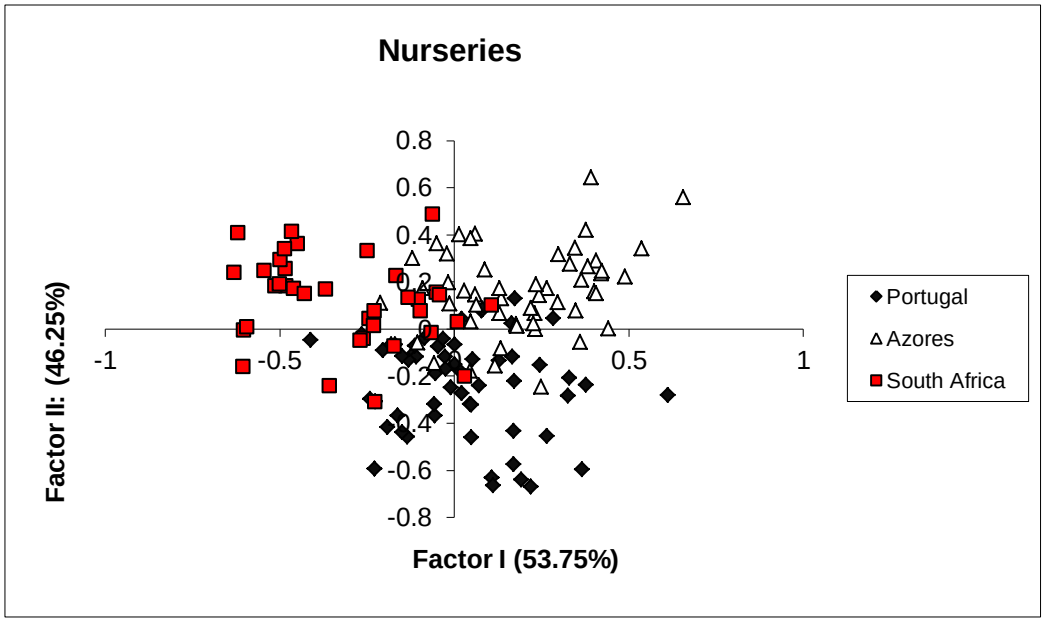

Figure 4. Factorial Correspondence Analysis (FCA) of multilocus microsatellite genotypes (13 loci) for *P. glauca* Atlantic nurseries.

Table 5. Levels of genetic diversity among nurseries of *P. glauca*, using nuclear microsatellite data. Pairwise F_{ST} including all 13 microsatellite loci below diagonal; and pairwise F_{ST} excluding Pgla03 above diagonal. **Bold**: *P*-values < 0.016 (after Bonferroni correction for multiple tests).

		F_{ST} excluding Pgla03		
		PT	Az	SA
F_{ST} overall loci	PT		0	0.0008
	Az	0		**0.0082**
	SA	0.0003	**0.0069**	

Table 6. Levels of genetic diversity among nurseries of *P. glauca* using the mtDNA CR sequences. Pairwise F_{ST} of mtDNA CR below the diagonal. **Bold**: *P*-values < 0.016 (after Bonferroni correction for multiple tests).

		PT	Az	SA
F_{ST}	PT			
	Az	0.0003		
	SA	0.0134	**0.0386**	

The results of the AMOVA analysis were not consistent between the nuclear microsatellite loci and the mtDNA CR (Table 7). The null hypothesis of panmixia among Atlantic blue shark nurseries was not rejected by the nuclear microsatellite data (F_{ST}: 0.002; *P*> 0.05). When the microsatellite locus Pgla03 was removed from the dataset, the same result was obtained ($F_{ST\ withoutPgla03:}$ 0.002; *P*> 0.05). Moreover most of the

genetic variation in the samples was found to be within individuals (i.e. 97.2%). In contrast, the null hypothesis of panmixia among Atlantic blue shark nurseries using mtDNA CR sequence data was rejected due to significant genetic heterogeneity among nurseries (F_{ST}: 0.016; ***P*< 0.05**).

The among-group component of genetic variance at the mtDNA CR was maximized by a structure of two groups of nurseries: South Atlantic (South Africa) vs. North Atlantic (Portugal and Azores) nurseries. Higher F_{ST} and F_{CT} values (including a F_{CT} *P*-value near significance) were obtained by the microsatellite loci for the same South/North Atlantic structure ($F_{CT\ overall\ loci:}$ 0.004; P> 0.05). Moreover, consistent results were obtained by the dataset of 12 loci (excluding Pgla03) and mtDNA CR sequences when considering the South vs. North structure of nurseries ($F_{CT\ withoutPgla03:}$ 0.005, $F_{CT\ mtDNA:}$ 0.027; **P≤ 0.05**). Contrarily, no significant genetic divergence was detected between oceanic (Azores) vs continental nurseries (South Africa and Portugal) ($F_{CT\ overall\ loci:}$ 0.001, $F_{CT\ withoutPgla03:}$ 0.001, $F_{CT\ mtDNA:}$ 0.004; P> 0.05) (Table 7).

Table 7. Analysis of molecular variance (AMOVA) for *P. glauca*. Fixation indices and respective *P*-value with, without the microsatellite locus Pgla03 and mitochondrial control region sequences.

Hypotheses	Nuclear Microsatellites (13)		Nuclear Microsatellites (12)		Mitochondrial CR sequences	
H0: Panmixia		*P*		*P*		*P*
F_{ST}	0.002	0.204	0.002	0.116	0.016	**0.043**
H0: Sa vs (Az+PT)						
F_{ST}	0.004	0.202	0.005	0.112	0.027	**0.041**
F_{SC}	-0.001	0.746	-0.001	0.654	0.000	0.391
F_{CT}	0.005	0.053	0.005	**0.050**	0.027	**0.000**
H0: Az vs (SA+PT)						
F_{ST}	0.002	0.206	0.003	0.112	0.017	**0.041**
F_{SC}	0.001	0.447	0.001	0.351	0.014	0.136
F_{CT}	0.001	0.340	0.001	0.309	0.004	0.333

Genetic Diversity of Atlantic Blue Shark Cohorts

Considering a cohort-based analysis, null alleles were detected by MicroChecker at the Pgla07 locus for the 2003 cohort, Pgla10 locus for the 2005 cohort, Pgla05, Pgla07 and TB15 loci for the 2006 cohort and for Pgla03 and Pgla07 for the 2007 cohort, due to an excess of homozygotes. Notwithstanding, all genotypic distributions were in accordance with HWE expectations for all locus/cohort combinations after Bonferroni correction (mean F_{IS} varied between -0.008 and 0.055; Table 10). The loci were not in linkage disequilibrium. The mean allelic richness (R_S) per cohort varied between 4.5 (2002 and 2004) and 4.9 (2003) (Table 8). Mean heterozygosities and R_S were similar among the sampled cohorts (H_O: 0.61-0.65; H_E:

0.59-0.67; R_S: 4.5-4.9). The cohorts of 2002 and 2004 had only 1 private allele (but had also the smallest sample size) while the cohort of 2006 presented the highest number of private alleles (8) (Table 8).

One to fourteen haplotypes of the mtDNA CR were found per cohort with half of the haplotypes (11) being shared by two or more cohorts. The cohorts of 2006 and 2007 revealed higher haplotype diversity (*h*= 0.91), and the 2007 cohort revealed also higher divergence between haplotypes (*k*= 2.78) as well as the highest number of private haplotypes (4), specially compared to the 2003 cohort (*h*= 0.77, *k*= 1.94) (Table 8).

Table 8. Genetic diversity indices of the blue shark, *P. glauca* gathering microsatellite loci and mtDNA CR haplotypes for each sampled cohort: number of genotyped or sequenced samples (N), mean number of alleles (Mean A), mean allelic richness (Mean R_S), range of allelic richness per locus (R_S range), mean observed heterozigosity (H_O), mean expected heterozigosity (H_E), fixation indices (F_{IS}) and Private Alleles; number of haplotypes (H), haplotype diversity (*h*), nucleotide diversity (π), the mean number of nucleotide differences between haplotypes (*k*) and private haplotypes (Private H). Note: the cohort of 2008 was excluded of this table because N=1.

	Nuclear Microsatellites								Mitochondrial CR sequences					
	N	Mean *A*	Mean Rs	Rs range	H_O	H_E	F_{IS}	Private Alleles	N	H	*h*	π	*k*	Private H
2002	8	4.7	4.5	2-11.7	0.58	0.60	0.109	1	7	4	0.81	0.0058	2.48	0
2003	22	7.5	4.9	1.8-11.3	0.65	0.65	0.013	6	18	6	0.77	0.0045	1.94	1
2004	11	5.4	4.5	1.6-11	0.61	0.59	0.011	1	10	6	0.89	0.0061	2.60	0
2005	39	8.2	4.6	1.8-11	0.63	0.62	-0.008	6	36	12	0.84	0.0062	2.64	1
2006	29	8	4.8	1.9-10.4	0.63	0.64	0.037	8	28	10	0.91	0.0062	2.64	3
2007	37	8.2	4.7	2.2-9.5	0.64	0.67	0.055	3	35	14	0.91	0.0065	2.78	4

Pairwise relatedness analysis also revealed that, within each cohort, the relatedness of individuals lies within the expected interval assuming random mating among individuals. The r index is also below or near 0 due to low relatedness of the individuals. Yet, two exceptions, the cohorts 2002 and 2004, exceed the tendency with a maximum upper limit of 0.015 that may be caused by small sample sizes of those cohorts (Fig. 5).

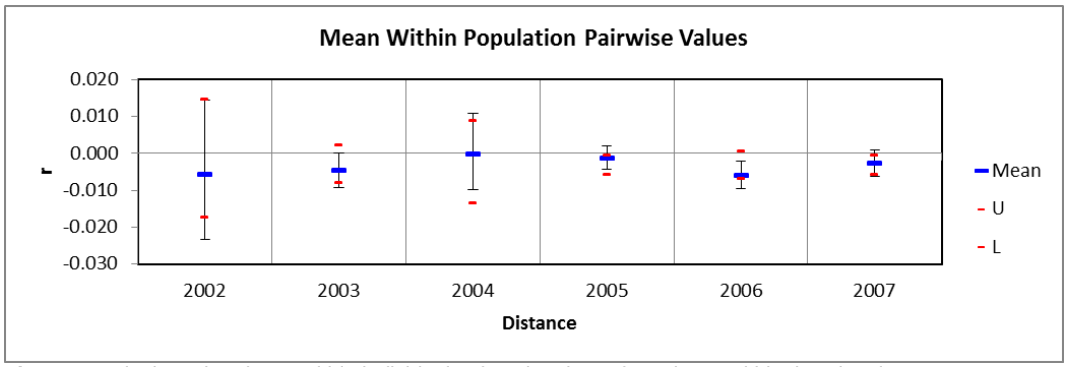

Figure 5. Pairwise relatedness within individuals of each cohort of *P. glauca* within the Altantic.

Genetic Differentiation among Atlantic Blue Shark Cohorts

The FCA on all the cohorts showed eigenvectors 1-3 explaining 68% of the inertia and revealed also separate distributions of multilocus microsatellite genotypes among the sampled cohorts and suggested that temporal genetic population structure may occur in Atlantic blue sharks (Figure 6). The PCA analysis showed a consistent separation of cohorts, however none of the axis had significant *P*-values (PC1: F_{ST} = 0.00537, 29% of total inertia, P = 0.699; PC2: F_{ST} = 0.00415, 23% of total inertia, P= 0.703).

Looking closely at the nurseries where more than one cohort was sampled e.g. off Portugal and off South Africa, (Figure 7 and 8), the same signal of temporal genetic structure is apparent. Within the Portuguese nursery, 70% of the inertia is explained by eigenvectors 1-3 (Figure 7), and there seems to be a separation of the 2002 and 2003 cohorts on the left, from the 2004, 2005 and 2006 cohorts on the right (Figure 7a). Notwithstanding, the PCA revealed non-significant axis (PC1: F_{ST} = 0.01638, 32% of total inertia, P = 0.383; PC2: F_{ST} = 0.01135, 22% of total inertia, P= 0.809). Within the South African nursery, eigenvector 1 explains 100% of the inertia and the distributions of multilocus microsatellite genotypes were completely separate between the two sampled cohorts, 2006 and 2007 (Figure 8). Yet again, the PCA revealed a non-significant axis (PC1: F_{ST} = 0.01286, 100% of total inertia, P = 0.614).

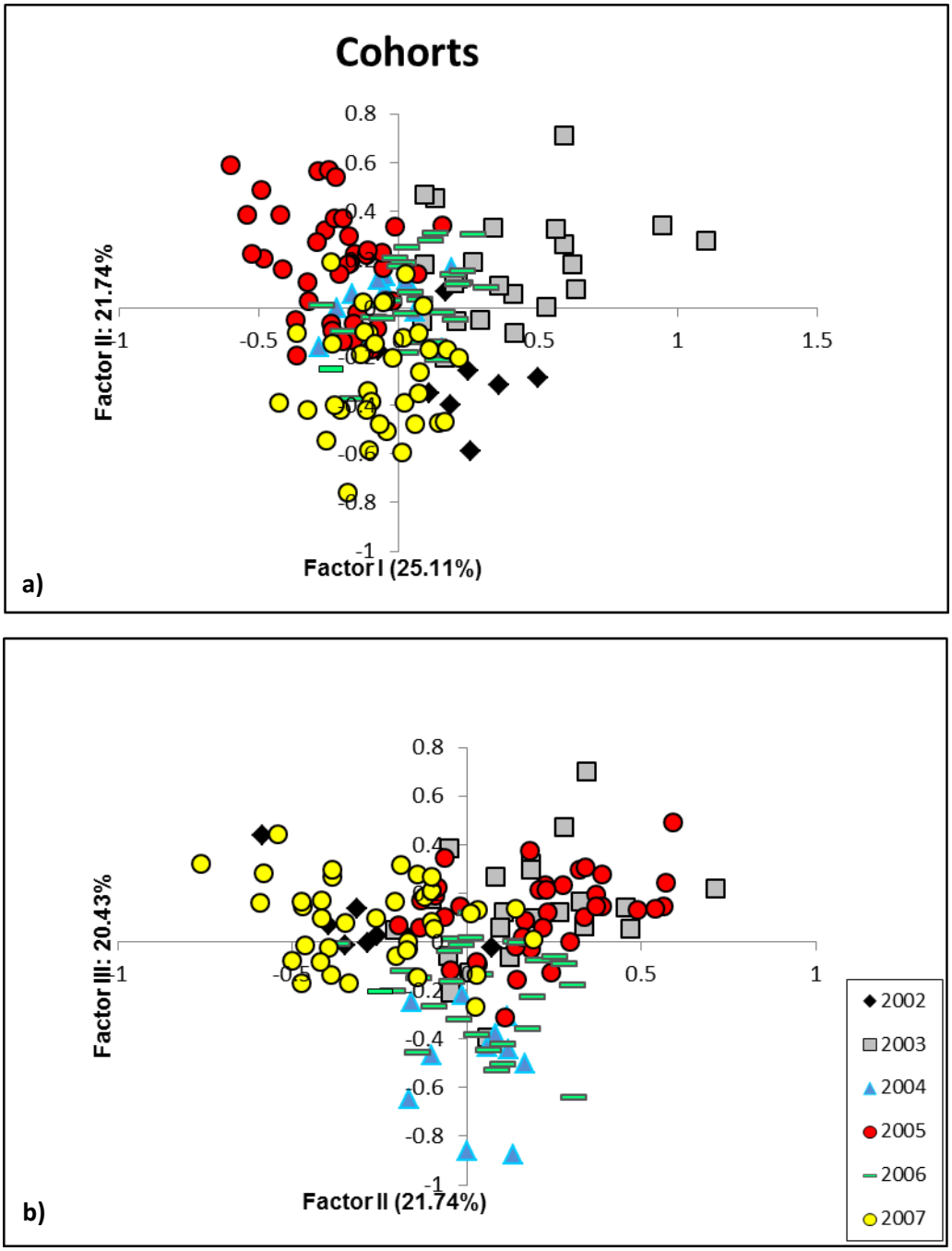

Figure 6. Factorial Correspondence Analysis (FCA) of multilocus microsatellite genotypes (13 loci) for six cohorts of *P. glauca*. Contibution of factors I and II a) and of factors II and III b) for the structuring.

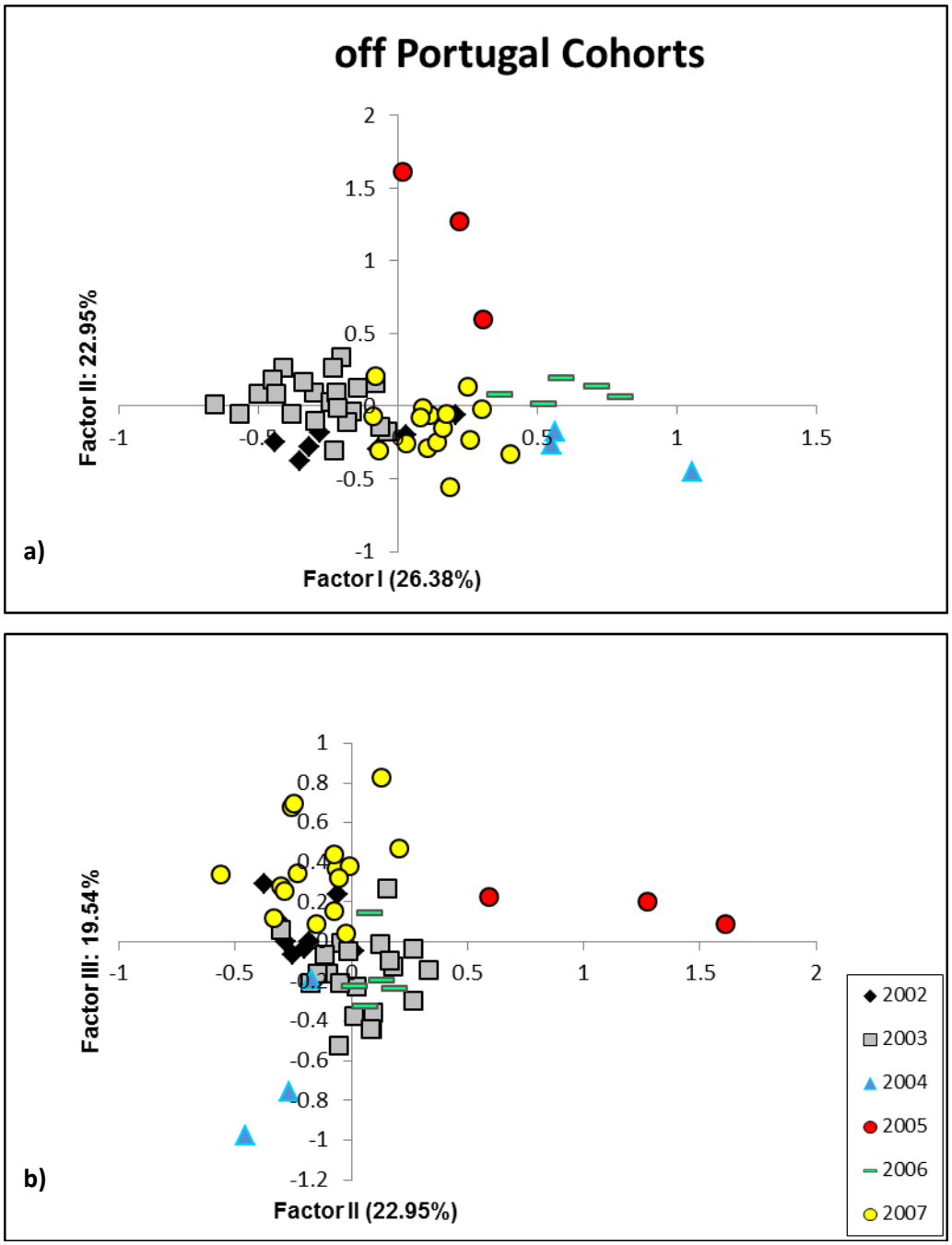

Figure 7. Factorial Correspondence Analysis (FCA) of multilocus microsatellite genotypes (13 loci) for six cohorts of *P. glauca* within Portugal nursery. Contribution of a) factors I and II and of b) factors II and III for the structuring.

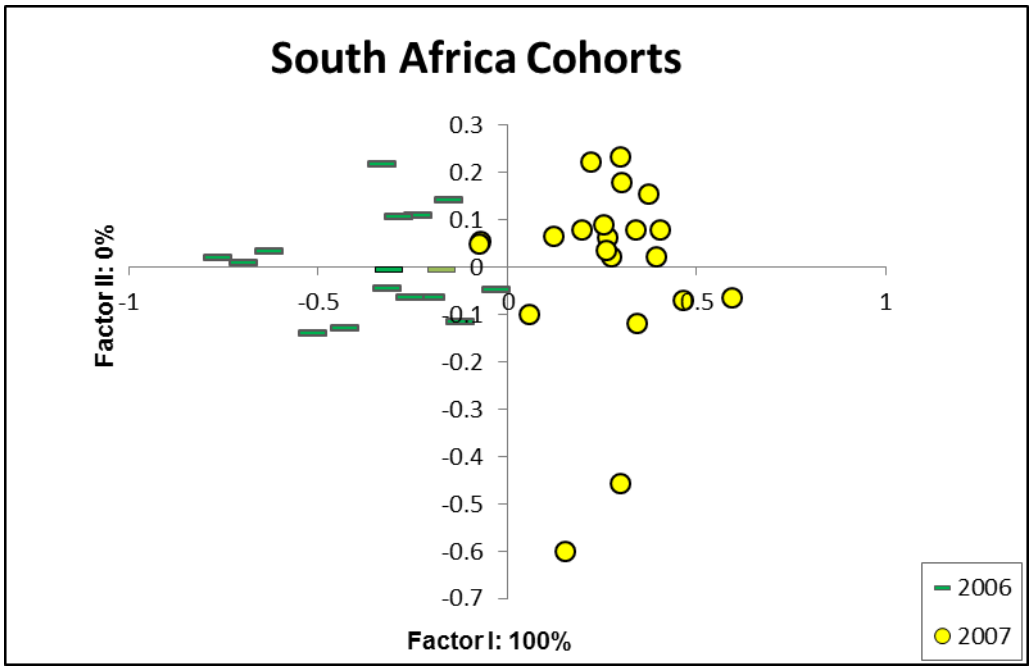

Figure 8. Factorial Correspondence Analysis (FCA) of multilocus microsatellite genotypes (13 loci) for two cohorts of *P. glauca* within South Africa nursery.

Pairwise F_{ST} tests among cohorts with >20 individuals each, indicated non-significant genetic differentiation between cohorts after Bonferroni correction (Table 9 and 10).

Table 9. Levels of genetic diversity among cohorts of *P. glauca* based on nuclear microsatellite data. Pairwise F_{ST} below the diagonal and pairwise F_{ST} above the diagonal without the microsatellite locus Pgla03. No *P*-values < 0.008 (after Bonferroni correction for multiple tests).

		FST excluding Pgla03			
		2003	2005	2006	2007
FST overall loci	2003		0.0038	0.0000	0.0029
	2005	0.0027		0.0014	0.0063
	2006	0.0000	0.0004		0.0000
	2007	0.0030	0.0058	0.0000	

Table 10. Levels of genetic diversity among cohorts of *P. glauca* Pairwise F_{ST} of mtDNA CR below the diagonal. No *P*-values < 0.008 (after Bonferroni correction for multiple tests).

		2003	2005	2006
FST	2003			
	2005	0.0437		
	2006	0.0301	0.0031	
	2007	0.0593	0.0489	0.0021

The results of the AMOVA analysis were consistent between nuclear microsatellite loci and mtDNA CR data. The null hypothesis of genetic homogeneity among Atlantic blue shark cohorts using both nuclear microsatellite data and mtDNA CR could not be rejected ($F_{ST\ overall\ loci}$: 0.002; $F_{ST\ without\ Pgla03}$: 0.002; $F_{STmtDNA}$: 0.010; $P>$ 0.05).

An FCA grouping nurseries and cohorts was also done. An overlapping of individuals occurs but some clusters of individuals with similar distributions of multilocus microsatellite genotypes are also observed. The clusters grouping seem to be based on differentiation among the sampled nurseries rather than among the sampled cohorts (Figure 9).

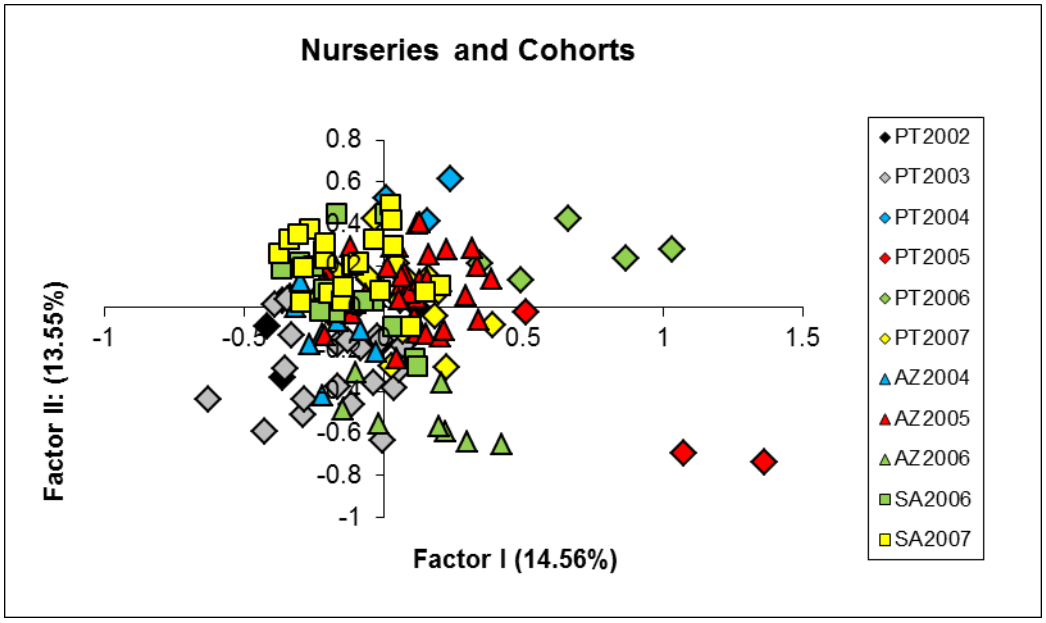

Figure 9. Factorial Correspondence Analysis (FCA) of multilocus microsatellite genotypes (13 loci) for *P. glauca* Atlantic nurseries and cohorts.

Discussion

Intraspecific Comparison

This study aimed to evaluate the genetic structure of the blue shark *Prionace glauca* within the Atlantic Ocean by assessing for the first time the genetic variation of small juvenile blue sharks sampled at reported nursery areas. The sampling strategy followed here aimed at targeting the most resident component of blue shark populations to avoid a confounding signal of genetic population structure among highly mobile adults from unknown origin. Additionally highly polymorphic molecular markers were chosen to evaluate the genetic diversity of the individuals. This strategy was selected to maximize the putative signal of genetic structure among nurseries.

Similar levels of genetic diversity were found by both marker types among blue shark nurseries in the Atlantic. Nevertheless, the nuclear microsatellites and the mitochondrial DNA CR sequences were able to detect genetic heterogeneity among juveniles sampled in distinct nurseries. Pairwise comparisons of genetic diversity showed low but significant genetic differentiation between Azores and South Africa, with both marker types. In contrast, the pairwise comparisons between Portugal and Azores and between Portugal and South Africa were low and not significant, though Portugal was genetically more similar to the Azores nursery than to the South African one. In line with the previous results, the AMOVA based on mtDNA data suggested the presence of two groups of blue sharks based on haplotype frequency differences: a North Atlantic group, including the Portuguese and Azorean nurseries, and a South Atlantic group, including the South African nursery. This structure maximized the genetic differentiation between geographically distinct nurseries, and is supported by distinct allelic frequencies and private alleles (North Atlantic: 21; South Atlantic: 6) at the microsatellite loci, and by different haplotype frequencies and private haplotypes (North Atlantic: 9; South Atlantic: 3) at the mitochondrial DNA level. The PCA analyses also supported the separation of North Atlantic (off Portugal and Azores) and South Atlantic (South Africa) nurseries based on allele frequency differences.

Nevertheless, within the North Atlantic, two genetic stocks (Portugal and Azores) may also be present as suggested by the FCA among nurseries, which compares the allelic composition across the 13 loci among all sampled individuals. The structuring among nurseries seen in the FCA may seem contradictory to the absence of genetic differences between the North Atlantic nurseries seen with F_{ST} tests and

AMOVA based on allelic frequency differences. However, this pattern can be explained by a non-random association of alleles at either the single locus or multilocus levels between samples in the absence of significant allelic frequency differences (Gillet 2013). Moreover, the high proportion of private haplotypes found in Portugal, which is roughly half of the total number of haplotypes on that nursery suggest some level of isolation from the remainder nurseries.

The null hypothesis of Atlantic panmixia in *P. glauca* was rejected by mtDNA CR nucleotide sequences but not by the nuclear microsatellite markers. The incongruity between markers in the rejection of panmixia may be caused by a lack of power of the microsatellites or by the absence of differentiation based on allelic frequencies among nurseries. One of the explanations most commonly proposed to explain the contrast between a signal of genetic structure at the maternally inherited mtDNA and its absence at bi-parentally inherited nuclear markers is the existence of female philopatry to nurseries in presence of male-mediated gene flow (Pardini et al., 2001; Portnoy et al., 2010; Tillett et al., 2012). However, in this study, the FCA result among nurseries does not support male-mediated gene flow, instead suggesting the existence of male "philopatry" to mating areas exclusively contributing to a single nursery ground. Independent evidence supporting this hypothesis refers to reports of blue shark male clubs (Litvinov, 2006), i.e. discrete aggregations on adult males within particular regions of the ocean. These "male clubs" are thought to function as areas to facilitate the meeting of males and females for further mating. The existence of discrete male clubs in addition to female philopatry to discrete nursery areas has been observed for the white shark (Jorgensen et al. 2010). Philopatric behavior of male and female blue sharks can result in genetic structure among nurseries within the Atlantic observed at different levels: multilocus genotypes of microsatellites and haplotypic frequencies of mitochondrial DNA.

Genetic population structure can occur both spatially and/or temporally, and a signal of spatial genetic structure may in fact be caused by temporal genetic heterogeneity not being adequately assessed in the samples. Random variation in allele frequencies among cohorts and sampling years are usual in species with overlapping generations (Jorde & Ryman, 1995), as the blue sharks. Indeed, despite the overall pattern of genetic divergence found here among nurseries of *P. glauca*, there was also a signal of temporal genetic structure in the samples. Exploratory FCAs suggest the existence of genetic heterogeneity among blue shark cohorts in the Atlantic Ocean, both across and within nurseries. However, there were several limitations in the current sampling strategy: the cohorts present in the overall sample were not equally represented in each nursery, and suffered also from small sample

sizes. The Azores nursery was mainly constituted by the 2005 cohort while South Africa had equal sample sizes of cohorts of 2006 and 2007. Portugal nursery had highly heterogeneous composition of cohorts, i.e. between 2002 and 2008. This has limited the power of the comparative analysis of temporal genetic differentiation among blue sharks cohorts. Namely, PCA analyses, pairwise F_{ST} tests, and AMOVA with both types of molecular markers did not detect significant genetic differentiation among blue shark cohorts, based on allele and haplotype frequency differences. The results presented are thus uniquely exploratory and warrant further confirmation. Thereby this study could not identify the cause of the signal of genetic structure of blue sharks within the Atlantic Ocean: geographical, temporal or a combination of both. In fact the FCA which considered both nurseries as well as cohorts can uniquely detect an influence of space.

In summary, significant genetic heterogeneity was found among Atlantic nurseries of blue sharks suggesting North Atlantic vs South Atlantic groups based on haplotype frequency differences. Moreover, there was evidence of further structuring between North Atlantic nurseries based on multilocus genotypes (e.g. FCA). In addition to geographical structure of blue sharks within the Atlantic, a signal of temporal genetic structure among blue shark cohorts across and within nurseries was also detected but needs further confirmation.

The separation of North vs South Atlantic nurseries of the blue shark based on haplotype frequency data is in accordance with results from a previous study, which showed evidence of a single panmictic population of blue shark in the North Atlantic (Queiroz, 2010). More recently a global study of genetic structure of blue sharks, using both nuclear and mitochondrial markers and targeting adults and subadults combined, suggests North and South genetic stocks separated by the Equator (Fitzpatrick, 2012). The results from genetic population studies are also in line with current assumptions of a North and a South stock within the Atlantic Ocean based on tag-recapture studies (Kohler et al., 1998; ICCAT, 2009). Of the total tagged blue sharks within the North Atlantic, only 1% was recaptured within the South Atlantic (ICCAT, 2009).

No other studies reported two stocks of blue sharks within the North Atlantic Ocean. The separation of Portugal and Azores in the FCA analysis among nurseries is unlikely to be random, and suggests the existence of differential association of alleles across the 13 loci between individuals from the different nurseries while the nonsignificant genetic differences based on F_{ST} tests between Portugal and Azores reflect the similar allelic frequencies at the microsatellite loci. However, limitations of F_{ST} estimates may be causing the absence of differentiation. Measures traditionally used to estimate genetic differentiation (e.g. F_{ST}, G_{ST} and relatives) based on allelic

frequencies may not reflect genetic differences (Gillet, 2013). Gillet (2013) demonstrates that two populations with the same number of equally frequent alleles, but in which no allele is shared between the two populations, will result in a F_{ST} value around 0.05 (i.e. close to zero) when in fact the two populations are completely different. Thus, low F_{ST} values may lead to undetected genetic differentiation when in fact it is present, as may occur with North Atlantic nurseries of blue sharks. To overcome these limitations other measurements of genetic differentiation like Jost's *D* (Jost, 2008) or compositional differentiation (i.e. recognition of gene associations; Gillet, 2013) should be considered in addition to the traditional ones.

Interspecific Comparison

Previous studies of genetic population structure of large, highly vagile and pelagic carcharhinid sharks, like the blue shark, generally found low levels of genetic differentiation both between and within oceans. In these studies, the genetic structure found between oceans is often explained by oceanic barriers to gene flow. Cosmopolitan species like the dusky shark *Carcharhinus obscurus* and the copper shark *C. brachyurus* mtDNA CR revealed genetic structure between oceans caused by large open ocean expanses or landmasses like the Isthmus of Panama (Benavides et al., 2011a; Benavides et al., 2011b). Genetic patterns at this level can also be caused by philopatry of females to nurseries located in different oceans. Carcharhinid sharks often exhibit this particular life-history trait that may help explain population structure, i.e. philopatry of females to nursery areas in which females regularly return to the same areas to give birth (Feldheim et al. 2001; Keeney et al. 2003). The blacktip shark *C. limbatus* and the sandbar shark *C. plumbeus* showed genetic structure at the mtDNA CR between oceans caused by female philopatry to nurseries (Keeney & Heist, 2006; Portnoy et al., 2010).

At the within-ocean level, genetic structure is mainly explained by the same philopatric behavior of females to nurseries. Carcharinid sharks like the blacktip shark *C. limbatus*, the sandbar shark *C. plumbeus*, the dusky shark *C. obscurus* and also the bull shark *C. leucas* present significant genetic structure within oceans at the mtDNA (Keeney & Heist, 2006; Portnoy et al., 2010; Benavides et al., 2011a; Karl et al., 2011; Tillett et al., 2012). *C. plumbeus* and *C. leucas* studies found also no significant genetic differences at microsatellite loci (Portnoy et al., 2010; Tillett et al., 2012).

Prionace glauca shares its evolutionary history with sharks of its own family, Carcharinidae, but shares the oceanic habitat with sharks of the Lamnidae family. Therefore it is interesting to compare blue shark genetic structure with patterns of genetic structure found in lamnid sharks, where open ocean expanses present no physical barriers to gene flow. Overall the studies suggest restricted gene flow associated to philopatry of females to nurseries in cosmopolitan and highly vagile oceanic species of the family Lamnidae (Jorgensen *et al*., 2010; Portnoy et al, 2010; Blower et al., 2012). The white shark *Carcharodon carcharias* mtDNA showed female philopatry within ocean basins and male-mediated gene flow between South Africa vs Australia and New Zealand, two populations within the Pacific Ocean and two around Australia (Pardini et al., 2001; Jorgensen et al., 2010; Blower et al., 2012). Another lamnid, the shortfin mako *Isurus oxyrinchus*, revealed significant structure at the mtDNA between and within Oceans and also at the microsatellite loci between oceans (Heist et al., 1996; Schrey & Heist, 2003).

Sharks of both Carcharhinidae and Lamnidae families have members with known female philopatry to nursery grounds. Philopatry of females is a cause of population structure within species and is the main explanation for the mtDNA genetic structure found within and between oceans. The results in the present study indicate the existence of genetic structure for the blue shark at the within ocean level. Inter-oceanic and intra-oceanic genetic structure has also been previously reported for the blue sharks (Queiroz, 2010; Fitzpatrick, 2012). In this study, female philopatry to either North or South Atlantic nurseries is suggested by significant genetic differentiation at the maternally-inherited mtDNA CR. However, as mentioned above, genetic differentiation at the microsatellite multilocus genotypes on the FCA supports blue shark structuring among Atlantic nurseries similar to the one reported for white sharks by telemetry (Jorgensen et al., 2010). Male white sharks were observed converging to small areas of about 250 km radius spatially structured (Jorgensen et al., 2010). Besides, temporal genetic structure of elasmobranchs was not detected on other studies but should be taken into account in the future.

Conservation and Management Implications

Knowledge of the population structure of a commercially exploited species is of crucial importance for management of fisheries. Therefore the establishment of stock boundaries is of utter importance for the study of demographic parameters like recruitment, growth and mortality (natural or fishery-induced) and the establishment of

fisheries´ management strategies, especially having into account that the extinction of a population is irreversible. An integrated understanding of the population structure of blue sharks is fundamental to achieve good management plans specially if considering the scenario of increased fishing effort in the North Atlantic.

Based on F_{ST} tests and the AMOVAs, two genetic stocks of blue sharks within the Atlantic Ocean were found in this study. The structure which maximizes genetic differentiation and is supported by allele and haplotype frequencies is that of North and South Atlantic genetic stocks of blue sharks. This structure is in accordance with data from tag-recapture studies (ICCAT, 2009) and other genetic studies (Queiroz 2010; Fitzpatrick, 2012). Both North and South Atlantic are characterized by cold productive waters divided by warm and poorly oxygenated waters at the Equator, which may function as a barrier to gene flow.

Notwithstanding, when looking into another level of genetic diversity like the multilocus genotypes, three genetic stocks of *Prionace glauca* appear to be present in the Atlantic Ocean with two genetic stocks (Portugal and Azores) co-occurring within the North Atlantic. Until now no other studies reported this stock structure of blue sharks. If a “lumper approach” is taken considering a single North Atlantic stock instead of two distinct population units, fishing effort may impact two stocks simultaneously without regarding putatively different demographics and vulnerability to fishing. Moreover, Waples (1998) stated that to rebuild an overfished stock within a short time frame, a large number of individuals have to migrate to that stock. This is particularly true in species with long generation times, like the blue shark.

Fishing effort within the North Atlantic varies in space and time (Queiroz, unpublished data). The distribution of stocks of blue sharks in the Atlantic is not known; therefore the real impact of fisheries on each stock is undetectable. That is why movement patterns occurring by sex, life stage and life-history features of blue shark within the Atlantic Ocean need to be clarified.

Overexploited populations that are spatially segregated and receive a low number of migrants from other populations may have a fast decline of stock size and survival. Segregated components of the population can be targeted differently depending on the distribution of fishing effort in time and place (Queiroz, 2010). Then enhanced fishing effort on segregated elements of the populations, like females and juveniles at nurseries, can have impacts over the whole population and over a large geographical scale. Nurseries are particularly susceptible because loss of juveniles can preclude the sustainability of fisheries of this species. A lower capacity to compensate juvenile mortality on nurseries can have long-term impacts on the sustainability of this species. In the Azorean nursery of blue sharks Aires-da-Silva (2008) realized that

juveniles and females were being intensively caught during Spring, when parturition takes place and young-of-year are present at the nursery ground.

P. glauca is a top predator that has a main role on the stable maintenance of marine ecosystems and some impacts can have major consequences on several species (Litvinov, 2006). Notwithstanding the finning regulation within E.U. waters, no other management measures have been considered for the blue shark. International regulations would be advisable for the conservation of a cosmopolitan oceanic species, like the blue shark, because in the case of population decline the effect of overfishing in one location can spread through other locations across the distribution range of the targeted stock (Baum et al., 2003). This asks for collaboration among international fisheries regulators to deliver a long-term sustainable management of blue sharks, especially of the eastern side of the Atlantic where two of the tree Atlantic nurseries are located. Independent management of each nursery should be implemented in order to protect the future generations of recruits. Healthy nurseries are fundamental for the maintenance of adult population sizes so the establishment of Marine Protected Areas (MPAs) incorporating nurseries of blue sharks within the Atlantic would prevent the decline of this species in future years. Additional protection measures of blue shark stocks may include fishery quotas, size limits or limits to by-catch of blue sharks in pelagic longline fisheries. Perhaps most importantly, because blue sharks are the main by-catch of other more productive target species, catch limits should be set based on the less productive stock in the fisheries.

Future Work

Given the limitation of the current sampling strategy, it was not possible to disentangle the signal of spatial genetic structure from the putative temporal signal in the data. Future studies should include a stratified sampling scheme including the same cohorts at the different nurseries in order to enhance the resolution of the signal of genetic population structure. The collection of reasonable sample sizes per cohort and per nursery and the use of additional molecular markers may also be advisable to increase the power in detecting genetic differentiation among sample collections.

Recommended integration of genetic techniques with other methodologies like tag-recapture methods and satellite telemetry would add valuable knowledge in the frequency of migrations to mating and nursery areas and on the patterns of habitat use in blue sharks. For example, to test female philopatry to a given nursery area or

connectivity among nurseries, adult female blue sharks can be electronically tagged at the reported nurseries during the parturition period and followed for >12 month periods.

Ultimately a sustainable exploitation of this marine resource is dependent on further research to confirm the number of management units within the Atlantic Ocean. Following the establishment of Atlantic blue shark management units, it will be important to study the biology, demography and movement patterns of blue sharks belonging to each unit, to better inform fishery management and conservation.

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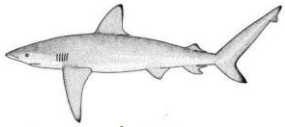
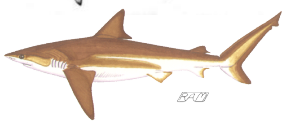
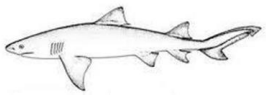
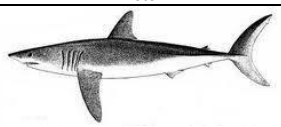
Annexes

I

Family	Species	Common name	Photo	Reference
Carcharhinidae	*Carcharhinus limbatus*	blacktip		Castro, 1993, 1996; Heupel & Hueter, 2002; Keeney et al., 2003; Keeney et al., 2005; Heist, 2005; Hueter et al., 2005; Keeney & Heist, 2006
	Carcharhinus plumbeus	sandbar		Castro, 1993; Heist et al, 1995; Heist & Gold, 1999; Merson & Pratt, 2001; Portnoy et al., 2007; Portnoy, 2008; Portnoy et al., 2010
	Carcharhinus leucas	bull		Karl et al., 2011; Tillett et al., 2012
	Carcharhinus obscurus	dusky		Benavides et al., 2011a
	Carcharhinus brachyurus	copper		Benavides et al., 2011b
	Rhizoprionodon terraenovae	sharpnose		Heist et al, 1996; Keeney & Heist, 2003
	Negaprion brevirostris	lemon		Feldheim et al, 2001; Feldheim et al, 2002; Feldheim et al., 2004; Schultz et al., 2008; DiBattista et al., 2008
	Galeocerdo cuvier	tiger		Keeney & Heist, 2003
	Prionace glauca	blue		Smith, 1986; Keeney & Heist, 2003; Queiroz et al., 2010; Fitzpatrick, 2012

Family	Species	Common name	Photo	Reference
Lamnidae	*Isurus oxyrinchus*	shortfin mako		Heist et al., 1996; Schrey & Heist, 2003
	Carcharodon carcharias	white		Pardini et al, 2001; Jorgensen et al., 2009; Blower et al., 2012
	Lamna nasus	porbeagle		Schrey & Heist, 2002
	Lamna ditropis	salmon		Schrey & Heist, 2002

Annex I: List of genetic studies for each species of the Elasmobranch families Carcharinidae and Lamnidae.

Annex II: *P. glauca* microsatellites amplified. **Locus:** marker name and **[primer]:** primer concentration on the PCR reaction of multiplex mix I and II.

	Locus	[primer] (µM)
MI	TB04F	0.032
	TB04R	0.32
	Pgla05F	0.032
	Pgla05R	0.32
	TB15F	0.056
	TB15R	0.56
	Pgla07F	0.04
	Pgla07R	0.4
MII	TB01F	0.04
	TB01R	0.4
	Pgla03F	0.008
	Pgla03R	0.08
	Pgla04F	0.008
	Pgla04R	0.08
	TB13F	0.04
	TB13R	0.4
	TB02F	0.028
	TB02R	0.28
	Pgla08F	0.001
	Pgla08R	0.1

Annex III: *P. glauca* haplotypes frequencies for each sampled cohort.

	Cohort							
Haplotype	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**Total**
Hap_1	2	8	2	7	5	5		29
Hap_2	3	3	2	12	4	5		29
Hap_3		1						1
Hap_4		3	3	4	4	3		17
Hap_5	1	2		3	4	5		15
Hap_6		1				1		2
Hap_7	1		1	1	3	7		13
Hap_8				1	1	1		3
Hap_9						1		1
Hap_10						1		1
Hap_11			1			1		2
Hap_12				2			1	3
Hap_13						1		1
Hap_14					1			1
Hap_15					2			2
Hap_16					1			1
Hap_17				1		2		3
Hap_18						1		1
Hap_19			1	1		1		3
Hap_20				2	3			5
Hap_21				1				1
Hap_22				1				1
Total	7	18	10	36	28	35	1	